The role of the Ubiquitin-Proteasome pathway in the pathogenesis of Amyotrophic Lateral Sclerosis

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ABSTRACT

Mutations in the Cu/Zn-superoxide dismutase (SOD-1) gene are responsible for a familial form of the motor neuron disease, amyotrophic lateral sclerosis (fALS). Aggregation of mutant SOD1 and formation of cytoplasmic inclusions are pathological hallmarks of the disease in patients and in transgenic mouse models of fALS, and their occurrence suggests overload of proteolytic and protein chaperoning pathways. We studied proteasomal function in transgenic mice over-expressing human SOD1 carrying the fALS-related mutation G93A ($SOD1^{G93A}$). Reduction in proteasomal activities in the lumbar spinal cord (containing vulnerable motor nuclei), but not thoracic spinal cord (less affected area) of pre-symptomatic $SOD\text{-}^{G93A}$ transgenic mice relative to non-transgenic littermates and $SOD\text{-}1^{WT}$ transgenic mice was measured early in disease pathogenesis. In addition, there was reduction of α-subunits of the 20S proteasome in lumbar spinal motor neurons relative to the surrounding neuropil with disease progression. Decreased proteasome activity in lumbar spinal cord corresponded with reduced levels of β3 (non-catalytic) and β5 (chymotrypsin-like activity) subunits of the 20S proteasome, despite normal levels of 20Sα subunits in the tissue as a whole. Even though levels of β3 and β5 were decreased, they were incorporated into 20S and 26S proteasome complexes as determined by native gel electrophoresis. Levels of β3 and β5 mRNA were not decreased in lumbar spinal cord of $SOD1^{G93A}$ transgenic mice prior to onset of symptoms, indicating unimpaired transcription and mRNA stability. Nor was there evidence that mutant SOD1 inhibited the proteasome by remaining associated with and blocking proteasomes. The relevance of findings in this mouse model of fALS to sporadic ALS was investigated by measuring proteasome activities and expression of proteasomal subunits in autopsy tissue from sporadic ALS patients As in fALS mice, proteasome activity was reduced in extracts of an affected region of spinal cord from ALS patients as compared to controls, whereas the proteasome activity in cerebellar tissue from these patients was not

affected. This impairment was coupled with a decrease of 20S β5 and 19S5a protein levels in spinal cord tissue suggesting that proteasome function is compromised in both familial and sporadic forms of ALS

RÉSUMÉ

Des mutations du gène de l'enzyme superoxyde dismutase Cu/Zn (SOD1) sont la cause d'une forme familiale de la sclérose latérale amyotrophique (SLAf), une maladie des neurones moteurs. L'aggrégation de la SOD1 mutée et la formation d'inclusions cytoplasmiques semblent être corrélées au développement de la maladie chez les patients et les modèles murins de la SLAf. Ces phénomènes suggèrent une surcharge des voies protéolytique et du chaperonnage protéiques. Nous avons étudié le mode de fonctionnement du protéasome de souris transgéniques surexprimant la SOD1 humaine portant la mutation liée à la SLAf, G93A ($SOD1^{G93A}$). Nous avons constaté que très tôt dans la pathogénèse de la maladie se produisait une réduction des activités protéasomales dans la moelle épinière de la région lombaire (ou se trouve les nucleus vulnérables des neurones moteurs) des souris transgéniques $SOD1^{G93A}$ pré-symptomatiques. Ce phénomène ne se produit pas dans la région thoracique de ces souris (une région moins touchée par la maladie), ni chez les souris non-transgéniques issues de cette même portée, ou portant la mutation $SOD\text{-}1^{WT}$. De plus, le nombre de sous-unités α du protéasome 20S dans les neurones moteurs de la moelle épinière de la région lombaire diminue au cours de la progression de la maladie, par rapport à ceux de la matière neuropil environnante. Une réduction de l'activité protéasomale dans la moelle épinière de la région lombaire coïncide à une diminution des taux des sous-unités β3 (non-catalytique) et β5 (activité type chymotrypsine) du protéasome 20S, bien que les taux globaux de sous-unités α dans le tissue soient normaux. Malgré la diminution des taux de β3 et β5, ceux-ci ont été isolés dans les complexes protéasomales 20S et 26S par électrophorèse native. De plus, les taux en ARN messager des sous-unités β3 et β5 ne diminuent pas dans la moelle épinière de la région lombaire des souris transgéniques $SOD1^{G93A}$ pré-symptomatiques, indiquant la préservation de la transcription et la stabilité de l'ARN messager. Aucun signe d'inhibition du protéasome par des protéines mutantes SOD1, que ce soit par association ou par blocage, n'a

pu être détecté. Pour démontrer l'importance de ces travaux pour l'étude de la SLA sporadique, l'activité protéasomale et l'expression de sous-unités protéasomales a été mesurée dans des tissus appartenant à des patients décédés de SLA. Comme pour les souris atteintes de SLAf, une réduction de l'activité protéasomale dans des extraits d'une région de la moelle épinière de patients atteints de SLA a été observée, en comparaison aux contrôles. Cependant, l'activité protéasomale dans les tissus cerebellaires de ces patients n'est pas affectée. Cette diminution d'activité est couplée à une baisse des taux en protéines 20S β5 et 19S 5a dans les tissus de moelle épinière, ce qui suggère une activité protéasomale limitée à la fois pour les formes familiales et sporadiques de la SLA.

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I would like to express my gratitude to everyone that helped through my PhD project (I do not think this page will suffice). First of all, thanks to my supervisor, Heather Durham who helped with her advice to overcome some of the problems that I faced as a young graduate student and for allowing me to transfer to the PhD program and completing these studies. A special thanks to Dr. Jeffrey Agar, who took so much of his time as a post-doctroral fellow in the laboratory to train me properly as a biochemist. Also, in a personal note, he was always available to listen and discuss every subject under the sun. Sandra Minotti, the technician in the laboratory was also essential to the animal work and was always ready and willing to make our life as easy as possible in the lab. I would like to thank also all past and present members of the lab, including almost Dr. Dave Taylor, Dr. Mehdi Doroudchi, Dr. Rebecca Aarons, Dr. Zarah Batulan, and Miranda Tradewell.

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It is hard to study a disease as horrible as ALS and not to think of the people suffering of this disease. I really hope that the advances in research and technology will continue to improve the quality of life for patients suffering from this disease as we come closer to finding a treatment for ALS. I cannot express all the gratitude to ALS Canada for their continuous support during my PhD studies and continuing their support by the generous offer of the Tim Noel post-doctoral award. Also, I would like to thank the Canadian Institute of Health Research for creating funding opportunities for students interested in ALS research.

I would also like my friends and family for their continuous and unconditional support. I want to thank my father, Kujtim, my mother, Irma and my brother, Sokol that even though they live 600 km away, they make me feel at home. Also, my family in Albania that is ready to welcome me with open arms whenever I go. Unfortunately, during my PhD studies three of my grand-parents passed away and it is to their memory that I would like to dedicate this thesis.

Finally, I want to thank Sorana Ciura for being the person that I shared everything for the last four years and I certainly hope for many more to come.

PREFACE

The thesis was written in the classical format according to guidelines by McGill University. The chapter that introduces mechanisms of ALS pathogenesis and the role of the ubiquitin-proteasome in this disease has been published previously in a review and a book chapter:

Kabashi E., Durham H.D. (2006) Failure of protein quality control in amyotrophic lateral sclerosis. Biochim Biophys Acta. June 18; (Epub ahead of print).

Durham H.D., **Kabashi E.**, Taylor D.M., Agar J.N. (2005) Motor neuron diseases: THE PROTEASOME/UDPS IN NEURODEGENERATIVE DISEASES AND AGING *Plenum US.*

Rationale and research objectives are presented in Chapter 2, followed by a materials and methods section in Chapter 3 that includes all procedures utilized in chapters describing results of this thesis. A brief introduction, general approach, results and discussion of these results are described in Chapters 4, 5 and 6. The results appear in the following articles that have been published or are in the submission process as indicated below:

Kabashi E., Agar J.N., Taylor D.M., Minotti S., Durham H.D. (2004) Focal dysfunction of the proteasome: a pathogenic factor in a mouse model of amyotrophic lateral sclerosis. Journal of Neurochemistry 89:1325-35.

Kabashi E., Agar J.N., Hong Y, Minotti S.M., Figlewicz D.A., and Durham H.D. Mechanism of proteasome dysfunction in a mouse model of Amyotrophic Lateral Sclerosis, To be submitted for review to Journal of Biological Chemistry..

Kabashi E., Agar J.N., Leystra-Lantz C, Strong M.J., and Durham H.D. Focal Impairment of the Proteasome in Spinal Cord of sporadic Amyotrophic Lateral Sclerosis patients. Manuscript in preparation

A general discussion containing a section with future directions is presented in Chapter 7. References are cited in Chapter 8. An appendix of supporting documents is located at the end of the thesis.

Throughout the PhD studies, I participated in several other projects and studies that are not covered in this thesis. Several of these studies have been published or are in the submission process as indicated below:

Taylor D.M., **Kabashi E.**, Agar J.N., Minotti S., Durham H.D. (2005) Proteasome activity or expression is not altered by activation of the heat shock transcription factor Hsf1 in cultured fibroblasts or myoblasts. Cell Stress Chaperones 10: 230-41.

Agar J.N., ,**Kabashi E.**, Gibbs, B.F.,.Bateman K.P., Taylor D.M., and Durham H.D. (2005) In Vivo Oxidative Modification of Superoxide Dismutase: Characterization by Mass Spectrometry. Manuscript in preparation.

Taylor D.M., Gibbs B.F., Aguiar M., **Kabashi E.**, Minotti S., Durham H.D., Agar J.N., - Tryptophan 32 is required for cytotoxicity and aggregation of a mutant superoxide dismutase (G93A) in familial amyotrophic lateral sclerosis. Submitted to Proc. Natl. Acad. Sci. USA

I carried out most experiments presented in this thesis with these notable exceptions. After designing the experiments, immunohistochemical analyses shown in Figures 3 and 10 were performed by David Taylor in Dr. Heather Durham`s laboratory. After designing the experiments and several of the primers used in the studies described in Figures 16 and 17, all rt-PCR experiments and statistical analysis of the data were carried out by Yu Hong in Dr. Denise Figlewicz`s laboratory. Dr. Jeffrey Agar and David Taylor carried out the experiments in Figure 20B and I participated in designing these experiments. These results were included in the thesis since the data presented are important in describing mechanisms of proteasome impairment in lumbar spinal cord tissue of $SOD1^{G93A}$ transgenic mice. As previously acknowledged, Dr. Jeffrey Agar, a post-doctoral fellow in Dr. Heather Durham`s laboratory was instrumental in assisting me with designing experiments, analyzing data, discussing ideas and participated actively in the experiments presented in Chapter 4. Sandra Minotti, technician in Heather Durham`s laboratory was in charge of maintaining the animal colony and removal of the spinal cord tissues used in the experiments in Chapter 4 and 5.

The members of my advisory committee, Dr Simon Wing and Dr. Ted Fon, two experts on the ubiquitin-proteasome pathway, were instrumental in discussing projects and the experimental approach taken during my PhD studies. Finally, Dr. Heather Durham provided all the scientific and financial support to complete the studies described in this thesis.

Other significant contributors include Sorana Ciura from Dr. Charles Bourque laboratory for continuous input and help with the project, Drs. Giovanna Pellecchia, Dr. Brad Kauffman and Dr. Florin Sassarman, post-doctoral fellows from Dr. Eric Shoubridge's laboratory at the Montreal Neurological Institute who provided a lot of technical support for Blue native gel electrophoresis and immunoprecipitation protocols. Dr. Marco DiFalco and Dr. Leonid Kriazhev at McGill University conducted mass spectrometric analysis of proteins from native gels. Dr. Vita Vernace from City University of New York and Dr. Daniel Finley from Harvard University helped immensely with protocols for native gel electrophoresis. Dr. Luke Szweda at the University of Oklahoma provided an antibody specific to HNE-modified proteins. Finally, all experiments presented in Chapter 6 were possible by the kind donation of banked tissue from sporadic ALS patients and control cases from Dr. Michael Strong. Cheryl Leystra-Lantz in Dr. Strong`s laboratory, was very helpful with methodology and shipping of the tissues.

CONTRIBUTION TO ORIGINAL KNOWLEDGE

Several mechanisms of disease pathogenesis have been described in ALS and the accumulating evidence points to ALS being a multifactorial disease. However, even though there are many initial causes of ALS, common downstream pathways could contribute to pathogenesis and provide targets for therapy. Ubiquitinated inclusion bodies in motor neurons of ALS patients are a pathological hallmark of this neurodegenerative disorder. In animal models of fALS there is evidence of protein misfolding and aggregation very early in disease pathogenesis in affected tissues by the disorder. An increase in ubiquitinated and misfolded proteins, mostly degraded by the proteasome complex, would suggest an overload and possible impairment of the proteolytic capacity of the proteasome may be a common phenomenon in ALS pathogenesis. In this thesis, the role that the ubiquitin-proteasome pathway plays in ALS was described.

Using a well-established mouse model of fALS, $SOD1^{G93A}$ transgenic mice it was demonstrated that the three major activities of the proteasome are reduced to about half of control activities early in the course of the disease, and specifically within the lumbar spinal cord, and that motor neuron-specific loss of proteasome expression occurred in $SOD1^{G93A}$ mice at the symptomatic stage. That proteasomal dysfunction occurred early and specifically in a region responsible for clinical symptoms indicates this property is important in the aetiology and progression of the disease. This was first time-course study of proteasomal function in a model of neurodegenerative disease and the first demonstration of either a focal decline of proteasome levels in a vulnerable cell-type or the impairment of the specific activity of the proteasome in an affected region as the disease progresses. These results are presented in Chapter 4 were previously published in Kabashi et al. 2004: *Focal dysfunction of the proteasome: a pathogenic factor in a mouse model of amyotrophic lateral sclerosis.* *Journal of Neurochemistry* *Vol. 89 p.1325-35.*

Analysis of proteasome structure and composition as well as the effect that mutant SOD1 exerts on the proteasome activity was performed to describe molecular mechanisms of proteasome impairment in lumbar spinal cord of $SOD1^{G93A}$. A specific decrease of constitutive 20S β subunits coincided with impairment of the proteasomal activity. These changes were not due to catalytic switches of β subunits to known immunoproteasome subunits, impaired transcription, or failure of these subunits to assemble in multimeric proteasomal complexes. These results are novel to the field of ALS research and this is the first study of proteasomal complex assembly in an animal model of neurodegeneration. These results are presented in Chapter 5 and a manuscript has been prepared for submission to *Journal of Biological Chemistry.*

Since fALS with SOD1 mutations accounts for only 2% of ALS patients, proteasome activity and its levels were determined in thoracic spinal cord tissue and a less affected tissue, cerebellum of ALS patients. Impairment of the three major activities of the proteasome were associated with decrease the functional constitutive β5 occurred solely in spinal cord tissue. These results are presented in Chapter 6 and are being prepared for publication.

The results presented in this thesis highlight the importance that the ubiquitin-proteasome pathway plays in ALS pathogenesis. In light of these experiments, therapeutic options that would help to specifically restore the impaired activity of the proteasome should be considered in ALS as well as in other neurodegenerative disorders where protein misfolding is a contributor of pathogenesis.

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LIST OF ABBREVIATIONS

ALS	amyotrophic lateral sclerosis (Lou Gehrig's disease)
ALS1	familial ALS linked to mutations in the gene encoding SOD1
ALS2	familial ALS linked to mutations in the gene encoding alsin
ALS-PDC	amyotrophic lateral sclerosis-parkinsonism-dementia complex
AMC	7-amino-4-methyl coumarin
AMPA	α-amino-5-hydroxy-3-methyl-4-isoxazole propionic acid
ARE	Anti-oxidant response element gene
ATP	adenosine triphosphate
BMAA	β-methylaminoalanine
Bcl-2	B-cell leukemia/lymphoma-2 anti-apoptotic protein
BSA	bovine serum albumin
C. elegans	Caenorhabditis elegans
CCS	Copper chaperone for SOD1
cDNA	complimentary DNA
CHIP	carboxyl terminus of Hsc/Hsp70-interacting protein
CNS	central nervous system
CNTF	ciliary neurotrophic factor
COX-2	cyclooxygenase-2
CSF	cerebrospinal fluid
DTT	dithiothreitol
DMSO	dimethyl sulfoxide
DNA	deoxyribonucleic acid
DRG	dorsal root ganglion/ganglia
EAAT2	excitatory amino acid transporter 2
EDTA	ethylenediaminetetraacetic acid
EMEM	Eagle's minimum essential medium
FALS	familial ALS
FDA	food and drug administration
GFAP	glial fibrillary acidic protein
GFP	green fluorescent protein
GluR2	glutamate receptor 2
HNE	4-hydroxy-2-nonenal
Hsc	heat shock cognate protein
HSE	heat shock element
Hsf1	heat shock transcription factor 1
Hsf2	heat shock transcription factor 2
Hsp	heat shock protein
HSP	family of Hsps
IGF-1	insulin-like growth factor-1
IgG	immunoglobulin G
IFNγ	Interferon γ
LCM	Laser capture microdissection
LM	Non-transgenic littermate
NF-H	neurofilament heavy
NF-L	neurofilament light
NO	nitric oxide
NOS	nitric oxide synthase(s)

Nrf2	nuclear factor, erythroid-derived 2, like 2
P	post-natal day
PBS	phosphate buffered saline
SBMA	spinal bulbar muscular atrophy (Kennedy's disease)
rt-PCR	Real-Time polymerase chain reaction
SD	standard deviation
SDS	sodium dodecyl sulfate
SDS-PAGE	sodium dodecyl sulfate polyacrylamide gel electrophoresis
SEM	standard error of the mean
SLA	sclérose latérale amyotrophique
SOD1	Cu/Zn-superoxide dismutase
$SOD1^{G37R}$	mutant SOD1 with glycine → arginine substitution at amino acid 37
$SOD1^{H46R/H48Q}$	double mutant SOD1 with histidine → arginine and histidine → glutamine substitutions at amino acids 46 and 48 respectively
$SOD1^{G85R}$	mutant SOD1 with glycine → arginine substitution at amino acid 85
$SOD1^{G86R}$	mutant SOD1 with glycine → arginine substitution at amino acid 86
$SOD1^{D90A}$	mutant SOD1 with aspartic acid → alanine substitution at amino acid 90
$SOD1^{G93A}$	mutant SOD1 with glycine → alanine substitution at amino acid 93
$SOD1^{L126Z}$	mutant SOD1 truncated after leucine at amino acid 126
$SOD1^{G127X}$	mutant SOD1 truncated after glycine at amino acid 127
t-BHQ	tert-butylhydroquinone
TNFα	Tumor necrosis factor α
UPS	ubiquitin-proteasome (proteolytic) system
Vsp54	Vacuolar-vesicular protein sorting gene
VEGF	vascular endothelial growth factor
WT	wild-type

Chapter 1 Introduction

Two highly important systems for maintaining protein quality control in cells, namely protein chaperones and the ubiquitin-proteasome pathway, are responsible for clearing damaged or misfolded proteins. Failure of these systems would contribute to the progression of amyotrophic lateral sclerosis (ALS), a neurodegenerative disorder that preferentially targets motor neurons. In this chapter, the current state of knowledge about ALS pathogenesis and the role that protein-quality control systems might play are reviewed.

1.1 Amyotrophic Lateral Sclerosis

1.1.1 Clinical Features of ALS

Amyotrophic lateral sclerosis (ALS) was first characterized by French physiologist Jean-Martin Charcot in 1869 (Mulder et al., 1986). The original diagnosis was based on neuropathological findings of degeneration of anterior horn cells and pathology of the corticospinal tract (Ince et al., 1998). The primary features of ALS include selective dysfunction and degeneration of upper and lower motor neurons (Cleveland and Rothstein, 2001). The term motor neuron disease, intending to encompass multiple idiopathic degenerative motor system diseases includes ALS, spinal bulbar muscular atrophy, progressive bulbar palsy, progressive muscular atrophy, and primary lateral sclerosis. Since ALS is the most common of these disorders, the term motor neuron disease is often used synonymously with ALS.

ALS, as described by Charcot, is a disease usually presenting in adulthood (around 50-60 years of age) as progressive muscle weakness and atrophy, muscle fasciculations and difficulties with speech and swallowing. The diagnosis of ALS is made in accordance with criteria established by the World Federation of Neurology Research Group on Neuromuscular

Diseases (Brooks, 1994). The predominant presenting symptom of ALS, muscle weakness, is due to the dysfunction and loss of motor neurons in the ventral horn of the spinal cord and brain stem that extend axons to synapse with muscle (Wang and Melhem, 2005b). In certain cases, there is also involvement of upper motor neurons in the motor cortex that synapse onto lower (brain stem and spinal) motor neurons (Bruijn et al., 2004). Depending upon which motor nuclei are involved, difficulty moving limbs and sustaining posture, speaking, swallowing and breathing may develop. Symptoms progressively spread within a region and to other regions of the neuromuscular system. As muscle fibres are denervated because of dysfunction or death of lower motor neurons, they may be re-innervated through sprouting of adjacent motor axons. Failure of re-innervation and decreased mobility result in muscle atrophy, giving a wasted appearance to the musculature.

The course of the disease can be devastatingly rapid, leading to progressive paralysis within the first year of clinical diagnosis. Failure of neurons that innervate the respiratory muscles usually results in the death of most ALS patients. The fatal event usually occurs within 3-5 years of clinical presentation (Cudkowicz et al., 2004).

1.1.2 Neuropathological Findings in ALS

Microscopic findings at autopsy include extensive loss of motor neurons, as well as reactive gliosis (both astrocytes and microglia) in the spinal cord and brain stem of patients. Surviving motor neurons present a number of ultrastructural abnormalities of surviving motor neurons including presence of ubiquitinated inclusions, neurofilament-rich hyaline inclusions, proximal axonal swellings filled with accumulations of neurofilaments, and dendritic atrophy (Hirano, 1982; Leigh et al., 1988; Murayama et al., 1989). Despite the selective vulnerability of motor neurons in ALS, other brain areas are not completely spared and in some cases may become sufficiently involved to manifest clinically, for example, neurons in the frontal cortex

controlling executive (planning) functions (Hasegawa et al., 1992; Strong et al., 2005). Interestingly, certain pools of motor neurons are spared in ALS, in particular those in the oculomotor and abducens nuclei that control eye movements (Okamoto et al., 1993) and those in Onuf's nucleus in the sacral spinal cord that control urinary and rectal sphincters (Schroder and Reske-Nielsen, 1984). All these characteristics have been reported in both sporadic and hereditary cases implying commonalities in pathogenesis regardless of the initiating cause.

ALS is a clinical syndrome with multiple underlying causes to which certain pools of motor neurons are particularly vulnerable. An important point is that multiple initiating factors (whether simple or complex genetic traits, environmental factors or a combination) manifest with similar pathology. The reaction of cells to the disease challenge may be just as important in their demise/survival as these causative factors and will depend on the other stresses to which they are subjected and the protective mechanisms at their disposal. Understanding these properties in motor neurons and the cells with which they interact provides the basis for design of therapeutic agents to prevent the cascade of events leading to cell death.

1.1.3 Epidemiology of ALS

The disease occurs with an incidence of 2/100,000 population or 3-4/100,000 over 20 yrs old, with a slightly higher affliction of women (male/female = 1.3/1.6:1) (Bobowick and Brody, 1973). The lifetime risk is about 1 in 2000 (Majoor-Krakauer et al., 2003). The ALS Society of Canada states that there are approximately 3000 people presently living with this disease in this country (refer to www.als.ca). The cause of most ALS cases is unknown, even though various genetic and environmental factors have been implicated (Shaw et al., 2001; Gros-Louis et al., 2006). Also the progression of the disease is quite variable suggesting that there might be a number of different factors involved. Among various environmental factors

to be associated with ALS, the toxin β-methylaminoalanine (BMAA), a potent excitotoxin of glutamate receptors found in the cycad fruit, common in the diet of people from the island of Guam, has received wide attention. Recently, a report has demonstrated biomagnification of BMAA in the flying fox, a dietary delicacy, that may have been associated with ALS/PDC (Cox et al., 2003). Attempts to correlate environmental or occupational exposures with ALS have identified clusters of ALS cases; for example a higher prevalence of ALS has been reported in professional soccer players in Italy (Chio et al., 2005) and in Gulf War veterans (Armon, 2004). However, the relatively small numbers of patients in most studies renders the statistical analysis unfeasible.

1.1.4 Genetics of ALS

Whereas some forms of ALS have a clear pattern of inheritance, 80-90% of cases remain of unknown cause, and thus are termed "sporadic". The hereditary form of the disease, familial ALS (fALS), is clinically and neuropathologically similar to sporadic ALS. In 1993, the first gene on chromosome 21 (ALS1) responsible for fALS was cloned and characterized (Rosen et al., 1993a). Over 100 mutations spanning all 5 SOD1 exons have been identified (see http://alsod1.iop.kcl.ac.uk) and are thought to cause disease because of a toxic gain of function. Most are missense mutations causing ALS in a dominantly inherited fashion with high penetrance, although a few are truncations. One missense mutation, D90A, exhibits a recessive or dominant pattern depending on genetic background (Jonsson et al., 2002).

Several other genes linked to motor neuron disease have been recently cloned and characterized. ALS2 causes a rare, juvenile-onset (3-23 year old) form of motor neuron disease and the gene was recently cloned (Hentati et al., 1994). Alsin, the gene product of ALS2, is a putative guanine exchange factor and has been implicated in organization of the actin cytoskeleton and vesicle trafficking and might have a role in maintaining motor neuron

survival (Jacquier et al., 2006). Surprisingly, two groups that generated alsin knockout mice did not report a neuronal phenotype and these mice had no developmental abnormalities (Devon et al., 2006; Hadano et al., 2006). Gene mutations in ALS4 (senataxin) (Chen et al., 2004) and ALS8 (vesicle-associated membrane protein B) (Nishimura et al., 2004) in fALS patients were recently described. However, these genes, similarly to ALS2, do not lead to dominantly inherited fALS, hence development of *in vivo* models using these genes might prove challenging. Gene defects in ALS3, ALS6 and ALS7 lead to classical dominantly inherited, adult onset fALS. Recently, loci have been identified for ALS3 on chromosome 18q (Hand et al., 2002), ALS6 on chromosome 16q (Abalkhail et al., 2003), and ALS7 on chromosome 20p (Sapp et al., 2003). Several groups are collaborating to map the regions and identify genetic mutations in these loci in order to develop new *in vitro* and *in vivo* models to study ALS pathogenesis (Gros-Louis et al., 2006).

1.1.5 Models of fALS

1.1.5.1 SOD1

The major finding that mutations in Cu/Zn superoxide dismutase (SOD1) causes fALS led to the development of various *in vivo* and *in vitro* models of ALS. SOD1 is an enzyme whose main function is to detoxify free radicals in the form of superoxide anions (O_2^-), by-products of mitochondrial oxidative phosphorylation, into water or hydrogen peroxide molecules, thus reducing oxidative stress on the cell. The reaction is a two-step process that involves reduction of SOD1 copper atoms by superoxide, followed by an oxidation step (Beckman and Koppenol, 1996). After 13 years of extensive study using these models, it still remains a mystery how mutations in the SOD1 gene lead to ALS. The loss-of-function hypothesis has not been convincing since knocking out the SOD1 gene in mice did not lead to neurodegeneration and most SOD1 mutants retain their dismutase activity (Borchelt et al.,

1994; Reaume et al., 1996). This evidence and the fact that fALS caused by SOD1 mutations is dominantly inherited indicates that a toxic gain-of-function of mutant SOD1 leads to motor neuron degeneration and ALS.

Indeed, toxicity of mutant SOD1 has been shown in various *in vitro* models, including motor neurons from spinal cord cultures, pure cultures of motor neurons, and neuronal cell lines (Durham et al., 1997; Menzies et al., 2002; Perrin et al., 2005). Overexpression of mutant SOD1 in motor neurons, but not in dorsal root ganglion (DRG) or hippocampal neurons led to formation of microscopically visible inclusion bodies and motor neuronal death (Durham et al., 1997). These *in vitro* models have proven valuable for studying mechanisms of disease pathogenesis and testing therapies to decrease mutant SOD1 aggregation and motor neuronal death.

The disease has been modeled in transgenic animals. Ubiquitous expression of various forms of mutant SOD1 in mice [human $SOD1^{G93A}$ (Gurney et al., 1994), human $SOD1^{G37R}$ (Wong et al., 1995), human $SOD1^{G85R}$ (Bruijn et al., 1997), human $SOD1^{L126Z}$ (Wang et al., 2005a), murine $SOD1^{G86R}$ (Ripps et al., 1995)] led to a neurodegenerative disorder associated with motor neuronal loss (see Table 1). Many other pathological features identified in spinal cord tissue from ALS patients, including ubiquitin-positive inclusion bodies in motor neurons and astrocytes, astrogliosis and microglial activation have been replicated in these murine models (Dal Canto and Gurney, 1995; Hall et al., 1998). Molecular and biochemical features, disease onset (usually indicated by hindlimb weakness and weight loss), as well as the progressive paralysis are remarkably reproducible within a particular strain of mice, providing a great tool for studying pathogenesis and testing therapeutics (Bendotti and Carri, 2004). The models described above, express very high levels of mutant SOD1. Recently, a mouse model of ALS has been generated expressing very low levels of truncated human $SOD1^{G127X}$ and could provide an alternative model to the lines described above (Jonsson et al., 2004).

Interestingly, this gene product has a higher propensity to aggregate than other SOD1 mutants even in tissue that is not affected (liver).

As seen in Table 1, most of these animal models have common features, such as loss of motor neurons at late stages of the disease, glial reactivity in spinal cord tissue and presence of ubiquitinated inclusions in motor neurons and astrocytes. Differences include mitochondrial vacuolation in motor neurons prominent in $SOD1^{G93A}$ early in disease pathogenesis but not in other lines, most likely due to high gene expression of mutant SOD1 (Dal Canto and Gurney, 1995). On the other hand, whereas formation of inclusion bodies is fairly rare in $SOD1^{G93A}$ transgenic mice, the line of mice carrying $SOD1^{G85R}$ have more prominent inclusions in spinal cord cells (Bruijn et al., 1997). These differences in murine models of fALS are important to consider since a mechanisms of disease pathogenesis might be more prominent in a certain line and might not model changes that occur in sporadic and fALS pathogenesis. Also, treatments that might prove very beneficial in one line might not change the progression of ALS pathogenesis in another line.

Recently, other animal models have been generated in order to better study ALS pathogenesis. *C.elegans* expressing mutant SOD1 have been very useful to study and visualize *in vivo* SOD1 aggregation (Oeda et al., 2001). Recently, two transgenic lines of rats overexpressing $SOD1^{G93A}$ and $SOD1^{H46R}$ were developed since larger tissue size (spinal cord) might help researchers understand mechanisms of ALS pathogenesis (Nagai et al., 2001; Howland et al., 2002).

1.1.5.2 Other Animal Models

Prior to cloning and characterization of SOD1 mutations, spontaneous mutations that would lead to selective degeneration of motor neurons were used as animal models of ALS. The spontaneous mutation that causes motor neuron pathology in one of the most popular of

these animal models, the wobbler mouse (Pioro and Mitsumoto, 1995) was recently characterized as a mutation in Vacuolar-vesicular protein sorting (Vps54) gene (Schmitt-John et al., 2005). The knowledge that a point mutation in the Vsp54 gene (the gene product plays a role in retrograde vesicular transport from the Golgi) leads to selective motor neuronal degeneration might open avenues of research to study molecular mechanisms of motor neuron pathogenesis in this model.

Several groups have also attempted to generate motor neuronal disorders by mutating genes to disrupt important molecular mechanisms of ALS pathogenesis in mice. Dynein, is a protein important in retrograde axonal transport along the microtubules (Ahmad et al., 1998). Expression of mutant dynein leads to disrupted axonal transport and selective degeneration of motor neurons with pathological features similar to murine models of fALS (*vide supra*) (Hafezparast et al., 2003). Vascular endothelial growth factor (VEGF) is important in motor neuron survival and treatment of lumbar spinal cord of $SOD1^{G93A}$ with VEGF delays the disease progression (see Section 1.3). Deleting the hypoxia inducible factor region of VEGF was demonstrated to lead to motor neuron degeneration and motor function abnormalities in mice (Oosthuyse et al., 2001). Peripherin is an intermediate filament up-regulated in neurons upon injury or inflammatory response (Troy et al., 1990). Overexpression of WT peripherin in mice provokes massive and selective degeneration of motor axons and motor neuron death by 6 months of age (Beaulieu et al., 1999). These mice provide the opportunity to identify common mechanisms of pathogenesis with different initiators of motor neuron disease and to possibly test the efficacy of pharmaceutical treatments that are successful in mutant SOD1 transgenic mice (see Therapeutical Perspectives).

1.2 Hypotheses of ALS pathogenesis

There is extensive evidence from studies of recombinant mutant SOD1 in vitro, from cell culture models and from transgenic animal models that a toxic gain of function conferred by disease-causing mutations of SOD1 leads to altered solubility of the protein and that insoluble proteins including mutant SOD1 collect into large, microscopically visible aggregates, although how this ultimately leads to death of motor neurons is not certain. However, mechanisms of mutant SOD1 misfolding and aggregation are becoming better understood (see Section 1.2.5.1). Various factors may contribute to motor neuron vulnerability are discussed below: increased oxidative stress; high level glutamatergic excitotoxic input and mitochondrial abnormalities; induction of the glial response; abnormalities in intermediate filament organization; and malfunction of the protein quality control systems. In a cellular context there is cross-talk between these molecular pathways as illustrated in Figure 1. Evidently, since ALS is a multifactorial disorder, all these mechanisms of pathology might occur simultaneously to varying degrees in each ALS patient and efficient treatments of ALS should take each one of these pathways into account.

1.2.1 Oxidative Stress

As is the case with each pathogenic mechanism described below, the major, unresolved debate remains whether increased oxidative stress is a primary cause of ALS pathogenesis or merely a consequence of this neurological disorder. Recent evidence as presented below demonstrates that in fALS1 animal models, oxidative stress is not the initiator of ALS pathogenesis but has a major contribution in disease progression through mechanisms that are independent of mutant SOD1 catalytic activity.

There is very good evidence for increased oxidative stress from studies in ALS patients and *in vivo* models of ALS (Barber et al., 2006). This includes increased protein carbonyl levels, increased 3-nitrotyrosine levels, and prominent immunolabeling for other

markers of protein and lipid oxidation specifically in affected areas of the spinal cord of ALS patients and mutant SOD1 transgenic mice as well as in affected cells (motor neurons, astroctes, microglia) (Shaw et al., 1995; Abe et al., 1995; Fitzmaurice et al., 1996; Beal et al., 1997). Also, increased oxidative damage to DNA as well as increased 4-hydroxynonenal (HNE) (indicative of lipid peroxidation), and ascorbate free radical levels have been reported in cerebrospinal fluid (CSF) samples from ALS patients (Fitzmaurice et al., 1996; Smith et al., 1998; Ihara et al., 2005). This hypothesis became much more persuasive in 1993, with the discovery that mutations of the anti-oxidant enzyme, SOD1, causes fALS (Rosen et al., 1993). However, a large body of evidence argues against mutant SOD1 causing oxidative stress by generating rather than detoxifying superoxide anions (refer to Section1.1.5).

Since copper plays an important role in SOD1 catalytic activity, other theories centered on the mishandling of active site copper in SOD1. Mutant SOD1 was shown *in vitro* to catalyze a backward reaction to produce more superoxide, which could combine with nitric oxide (NO) to form peroxynitrite, a potent protein-damaging agent (Beckman, 1994). This *in vitro* data correlated well with discoveries of increased NO synthases (NOS) and 3-nitrotyrosine in $SOD1^{G93A}$ (Bruijn et al., 1997; Ferrante et al., 1997; Almer et al., 1999; Cha et al., 2000; Sasaki et al., 2002) and human patients (Beal et al., 1997; Tohgi et al., 1999; Phul et al., 2000; Anneser et al., 2001; Sasaki et al., 2001; Catania et al., 2001). However, this mechanism does not appear to be crucial to ALS pathogenesis since inhibition of neuronal NO synthase (NOS) had no effect on survival of mutant SOD1 transgenic mice and cultured motor neurons (Facchinetti et al., 1999; Doroudchi et al., 2001).

Two *in vivo* studies were instrumental in determining that fALS caused by mutant SOD1 in transgenic mice is copper independent. Crossing several lines of mutant SOD1 transgenic mice with mice that lack the copper chaperone for SOD1 (CCS) did not alter disease progression (Subramaniam et al., 2002). To follow up, Borchelt and colleagues

combined disease-causing SOD1 mutations, $SOD1^{H46R/H48Q}$ with an inability to bind copper in transgenic mice and found no change in survival or motor neuron degeneration (Wang et al., 2003).

All this evidence arguing against the original hypothesis that mutation in SOD1 resulted in SOD1 that generated, rather than detoxified radicals, does not exclude the fact that mutant SOD1 may induce oxidative stress in a mechanism beyond its own catalytic activity. Recently, expression of mutant SOD1 in cell lines lowered by 3-fold the expression of an important anti-oxidant factor, Nrf2, a transcription factor that regulates the expression of anti-oxidant response element (ARE) genes (Kirby et al., 2005). This transcription factor is quite important because it regulates various "programmed cell life" genes as well as the expression of various proteasomal subunits (*see Section 1.2.6.4*). Also, as it will be discussed proteins severely damaged by oxidative stress, including mutant SOD1, may be powerful inhibitors of the proteasome activity (refer to Section 8.3).

1.2.2 High Level Glutamatergic input and Mitochondrial Abnormalities

1.2.2.1 Glutamatergic Excitotoxicity

Motor neurons are very large cells that are highly excitable because they express abundant levels of glutamate receptors and highly vulnerable to glutamate toxicity due to their reduced protective mechanisms. For this reason, excitoxicity, neuronal damage due to over-stimulation of glutamate receptors and elevation of intracellular Ca^{2+} levels by Ca^{2+}-permeable glutamate receptors, has an important role in ALS pathogenesis (Van Den et al., 2006). In motor neurons derived from primary dissociated and organotypic cultures, excitotoxicity is largely mediated by excitation of AMPA receptors as in other CNS neurons (Rothstein et al., 1993; Carriedo et al., 1996; Roy et al., 1998). Several lines of evidence support the involvement of glutamate receptor excitation in ALS including increased

glutamate levels in CSF of some, but not all ALS patients (Plaitakis et al., 1988; Perry et al., 1990). Pronounced loss of the glial glutamate transporter, EAAT2, responsible for clearing glutamate from the synaptic cleft, was measured in affected brain regions of ALS patients and in spinal cord of mutant SOD1 transgenic mice (Rothstein et al., 1995; Sasaki et al., 2000). Also, several studies have demonstrated that expression of several mutant SOD1 can impair *in vivo* glial glutamate transport in *Xenopus* oocytes (Trotti et al., 1999), therefore reducing the clearance of excess glutamate from around motor neurons by supporting glia.

Excitation of Ca^{2+}-permeable AMPA receptors appear to be another important factor in ALS pathogenesis since joro-spider toxin, an inhibitor of Ca^{2+}-permeable AMPA receptor preserved viability of motor neurons injected with mutant SOD1 and reduced mutant SOD1-specific aggregate formation (Roy et al., 1998; Kruman et al., 1999). Also, transgenic mice with increased Ca^{2+} permeability of glutamate receptors due to expression of GluR-B(N)-containing AMPA receptors had a late-onset motor neuron pathology independent of mutant SOD1 expression (Brusa et al., 1995). Finally, motor neurons express low levels of Ca^{2+}-buffering proteins, whereas less vulnerable motor neurons such as those in the oculomotor, trochlear, abducens and Onuf's nucleus clearly express higher levels of tCa^{2+}-binding proteins (Ince et al., 1993; Alexianu et al., 1994; Elliott and Snider, 1995; Reiner et al., 1995). Increasing resistance to calcium-mediated toxicity by overexpression of the Ca^{2+}-buffering proteins, calbindin-D28K in cultured motor neurons reduced motor neuron death and mutant SOD1 positive inclusion body formation (Roy et al., 1998). Also, cross-breeding of parvalbumin-overexpressing mice with $SOD1^{G93A}$ transgenic mice, significantly prolonged survival and onset of disease (Beers et al., 2001).

1.2.2.2 Mitochondrial Dysfunction

As illustrated in Figure 1, elevated intracellular Ca^{2+} levels would cause mitochondrial abnormalities and activation of cell death pathways since mitochondria are a major organelle responsible for buffering cytosolic Ca^{2+}. Also, since reactive oxygen species are generated in the mitochondrial respiratory chain, elevated levels of oxidative stress can further damage mitochondria. As mentioned previously, these mechanisms of ALS pathogenesis are certainly not independent entities but seem to act in concert.

Mitochondrial abnormalities have been identified in post-mortem muscle and spinal cord of sporadic and fALS patients (Fujita et al., 1996; Browne et al., 1998). Mitochondrial vacuolation in motor neurons and a focal decrease of various mitochondrial electron transfer chain complexes activity in spinal cord is an early event in mutant SOD1 pathogenesis of $SOD1^{G93A}$ transgenic mice (Dal Canto and Gurney, 1995; Higgins et al., 2002). However, this phenomenon might be an artifact of very high levels of mutant SOD1 expression in these mice since motor neurons from other mutant SOD1 transgenic lines did not have a pronounced mitochondrial vacuolation. Recent evidence suggests that mitochondrial dysfunction might play a more central role in ALS pathogenesis (Manfredi and Xu, 2005). Liu et al. demonstrated that in spinal cord tissue from mutant SOD1 transgenic mice, SOD1 progressively accumulates and aggregates in the outer mitochondrial membrane, causing clogging of the protein translocation machinery, and eventually resulting in mitochondrial dysfunction (Liu et al., 2004). Studies from yeast propose that some SOD1 is imported into the mitochondria where it is folded, metallated and becomes enzymatically active. Mutant SOD1 may fail to fold properly and to form the intracellular disulfide bond and clogs the import machinery of mitochondria (Culotta et al., 2006). There is some *in vivo* evidence to support this mechanism. Pasinelli and colleagues recently demonstrated that an interaction of Bcl-2 with mutant SOD1 in pre-symptomatic spinal cord tissue of $SOD1^{G93A}$ led to its sequestration in high molecular weight inclusions at the mitochondrial surface, which likely

depleted its antiapoptotic function contributing to disease pathogenesis (Pasinelli et al., 2004). However, the contribution of intramitochondrial mutant SOD1 to disease remains controversial since recent reports demonstrate that high hSOD1 expression rates can cause artificial loading of mitochondria and abnormalities (Bergemalm et al., 2006) that might not occur in animal models of fALS caused by a lower expression of mutant SOD1 (also refer to Section 1.1.5.1)

1.2.3 The Glial Response in ALS

1.2.3.1 Implications of Cellular Neighborhood

Two independent studies demonstrated that transgenic animals expressing mutant SOD1 driven by the neuronal promoters of NF-L (Pramatarova et al., 2001) or Thy1.2 (Lino et al., 2002), or by the astrocytic promoter GFAP (Gong et al., 2000) failed to develop motor neuronal disease and lacked developmental abnormalities. These studies strongly indicate that interplay between motor neurons and other cells in the region might be essential for ALS pathogenesis. Further evidence of this multicellular involvement came from a study where chimeric mice were composed of both normal cells and cells expressing mutant SOD1 in different mixtures in the spinal cord. This study demonstrated that motor neurons expressing high levels of mutant SOD1 survive much longer when surrounded by a wild-type glial environment than when mutant SOD1 is ubiquitously expressed. Recently, selective inactivation of the mutant SOD1 gene in microglia and motor neurons of $SOD1^{G37R}$ mice with Cre-Lox recombination demonstrated that diminishing mutant SOD1 in motor neurons slowed down the onset of the disease but did not affect significantly its progression, whereas reduction of mutant SOD1 specifically in microglia did not affect disease onset, but slowed down the disease progression (Boillee et al., 2006). These results led the authors to propose that the initiating phase of fALS1 is caused by mutant SOD1 damage within motor neurons,

and a mechanistically divergent later phase encompassing the progression to complete paralysis is linked to the inflammatory response of microglia and mutant toxicity within these cells.

1.2.3.2 Microglia and the Inflammatory response

These studies underline the importance of the glial response in ALS disease progression. Astrocytes participate by clearing glutamate levels in the synaptic cleft and releasing trophic factors, but when astrocytes become reactive, a phase characterized by high levels of GFAP expression, they can damage neurons (Barbeito et al., 2004). However, as indicated above, the most important players in the glial response are microglia. Damage to the CNS activates microglia which release cytotoxic and inflammatory substances that further damage neuronal cells. Dying motor neurons can release substances that activate microglia which in turn release cytotoxic and inflammatory substances causing a further augumentation of neuronal damage (Bruijn et al., 2004). Microglial and astrocytic activation and proliferation have been demonstrated in spinal cord of ALS patients and are an early event in mutant SOD1 pathogenesis in lumbar spinal cord of transgenic mice (Figure 4) (Alexianu et al., 2001; Sargsyan et al., 2005). Also, levels of pro-inflammatory factors (TNFα, IFNγ, COX2) are highly up-regulated early and specifically in spinal cord tissue of $SOD1^{G93A}$ transgenic mice (Hensley et al., 2003). The glial response and the resulting neuroinflammation might contribute to fALS pathogenesis and pharmaceutical treatments to reduce this response are currently being investigated (Refer to Section 1.3 and Table 2).

1.2.4 Abnormalities in Intermediate Filaments Organization

Proper neurofilament (NF) organization is essential for axonal transport and motor neuronal homeostasis. Abnormal organization resulting in NF accumulation is a common

pathological hallmark in affected tissue from sporadic and fALS patients (Carpenter, 1968; Hirano et al., 1984). Further research highlighted the important role of NF organization in ALS pathogenesis including discovery of deletion and insertion mutations in the heavy NF (NF-H) gene in sporadic ALS patients (Figlewicz et al., 1994; Tomkins et al., 1998) suggested that disruption of normal NF function is an important part of ALS pathogenesis. This theory was supported by experiments in which overexpression of human NF-H subunits led to a six-month extension of lifespan in an $SOD1^{G37R}$ model. (Couillard-Despres et al., 1998). Also, dominant mutation in the light NF (NF-L) genes are a primary cause of the motor neuropathy in Charcot-Marie-Tooth disease (Type II) (De Jonghe et al., 2001). These studies highlight that proper NF assembly is a contributor as well as a probable risk factor for ALS (Bruijn et al., 2004). However, a recent study failed to link NF mutations to sporadic and/or fALS, which implied that NF assembly and organization might not be a significant primary cause of ALS (Garcia et al., 2006).

Peripherin, an intermediate filament protein expressed during spinal motor neuronal development also may contribute to ALS pathogenesis (Robertson et al., 2002). Peripherin and NF expression were detected in the majority of axonal inclusions of ALS patients (Corbo and Hays, 1992). Overexpression of a splice variant, peripherin 58, in motor neurons was found toxic in transgenic mice (Beaulieu et al., 1999). Also, expression of peripherin 61 at low levels in primary cultured motor neurons was found to be highly toxic (Robertson et al., 2003). Peripherin 61 was also detected in lumbar spinal cord of ALS patients but not in controls (Robertson et al., 2003). Frameshift mutations as well as a missense mutation of the peripherin gene have been discovered in a few sporadic ALS patients (Gros-Louis et al., 2004; Leung et al., 2004). However, since eliminating or overexpressing peripherin did not change the pathophysiological course of the disease in $SOD1^{G37R}$ mice, its role in disease pathogenesis has come under question (Lariviere et al., 2003). Further studies are required to

clarify the contribution of specific splice variants and the factors that promote production of toxic isoforms.

1.2.5 Protein Misfolding in ALS

Many reports have demonstrated that genetic mutations and post-translational modifications cause proteins to misfold. In neurodegenerative disorders as well as physiological aging, protein misfolding is enhanced. Accumulating evidence demonstrates that cellular failure to clear these abnormal proteins might be a determining step in ALS pathogenesis. Research is striving to better understand the pathogenic proteins that might trigger disease, the events that might contribute to misfolded protein accumulation, as well as the possibility of enhancing the physiological responses of neurons to rid themselves of misfolded proteins.

1.2.5.1 Evidence of Protein Insolubility and Aggregation

Whereas $SOD1^{WT}$ is for the most part a soluble enzyme, disease-causing mutant proteins can be isolated in three different biochemical states: soluble in nonionic detergent, nonionic detergent-insoluble/SDS-soluble (up to 10%), and SDS-insoluble polymers (see Figure 6) (less than 0.5%) (Johnston et al., 2000), (Shinder et al., 2001) and (Wang et al., 2002). Large aggregates can be trapped by passing tissue homogenates through filter traps (Wang et al., 2002) and visualized as inclusions by immunohistochemical labeling of tissue sections or transfected cells (Shibata et al., 1996b), (Bruijn et al., 1997), (Wang et al., 2002) and (Watanabe et al., 2001). In transgenic mice, the amount of insoluble and high molecular weight mutant SOD1 is highest in tissue most vulnerable to disease, suggesting that cells in these regions process the mutant protein differently or inefficiently (Johnston et al., 2000), (Wang et al., 2002), (Kabashi et al., 2004) and (Wang et al., 2005a). Similarly, the propensity

of mutant SOD1 to form aggregates visible by microscopy depends on the cell type. The selective vulnerability of motor neurons manifests itself in culture, mutant SOD1 forming inclusions in motor neurons of spinal cord cultures following gene transfer, but not in dorsal root ganglion or hippocampal neurons (Durham et al., 1997). In various cell lines, treatment with proteasomal inhibitors (Johnston et al., 2000) promotes the formation of these inclusion bodies. Even mutant SOD1 that is immunolabeled diffusely may be in the form of small oligomers rather than the normal dimeric structure of $SOD1^{WT}$ (Matsumoto et al., 2005), consistent with a significant fraction of the protein being detergent-insoluble even in the absence of distinct inclusions (Shinder et al., 2001). This evidence points to the essential role that protein insolubility and aggregation plays in the pathogenesis of ALS.

Whereas there is evidence that inclusions may protect cells from accumulated misfolded protein rather than being inherently toxic, they do reflect failure of the proteolytic system to rid cells of potentially harmful proteins (Kopito, 2000). In the case of mutant SOD1, there are many studies in which inclusions served as a marker of toxicity, although this by no means demonstrates they are the cause of toxicity: the load of protein aggregates correlates with motor neuron dysfunction in a transgenic mouse model (Wang et al., 2005a); cultured motor neurons with inclusions develop DNA fragmentation and chromatin condensation (Durham et al., 1997); loss of viability correlates with formation of mutant SOD1 inclusions (Durham et al., 1997), (Johnston et al., 2000), (Matsumoto et al., 2005), and (Ghadge et al., 2006), and treatments that reduced formation of inclusions prolonged viability of mutant SOD1-expressing motor neurons (Bruening et al., 1999) and (Durham et al., 1997) and neuro-2A cells (Takeuchi et al., 2002). On the other hand, survival of differentiated PC12 cells transduced with mutant SOD1-YFP adenoviral vectors did not correlate with inclusions (however, aggregation of $SOD1^{WT}$ was also observed in the study) (Lee et al., 2002), and targeting mutant SOD1 to mitochondria induced cell death in the absence of cytoplasmic

inclusions, although oligomerization of mutant SOD1 in mitochondria was not excluded (Takeuchi et al., 2002). This is a complex issue that is influenced by the experimental paradigm (e.g., culture versus animal models, tagged versus untagged constructs, stable versus transient expression, exposure to additional stresses). Another issue that requires a lot of attention is which of these abnormal SOD1 forms (insoluble high-molecular weight species, filter-trap aggregates, proteolytic fragments, oligomers) is the most toxic to motor neurons.

Application of more sensitive techniques to resolve smaller oligomeric forms of mutant protein in cells and tissues is needed in order to visualize the process of oligomerization during ALS progression, since these are the species considered most likely to be toxic in cell lines (Mukai et al., 2005).

1.2.5.2 Post-Translation Modifications

Evidence points to a "toxic gain of function" conferring disease properties, with conformational abnormalities as the common property. Over 100 mutations in SOD1 cause disease, but have variable effects on enzymatic activity, and overexpression of $SOD1^{WT}$ failed to cause ALS-like disease (Cleveland and Rothstein, 2001) and (Bruijn et al., 2004). Mutant SOD1 proteins in cells are catabolized by the proteasome (Hoffman et al., 1996), (Johnston et al., 2000), (Niwa et al., 2002) and (Puttaparthi et al., 2003), and recombinant SOD1 mutants are good substrates for the 20S proteasome *in vitro*, particularly as metal deficient monomers lacking the intrasubunit disulfide bond (Di Noto et al., 2005).

Studies of recombinant proteins *in vitro* or transfected cultured cells subjected to oxidizing conditions show that dimer destabilization (SOD1 functions as a homodimer) and polymerization of monomeric SOD1 are promoted by disease-causing mutations, demetallation, disulphide reduction, and oxidative modifications (Cardoso et al., 2002),

(Rakhit et al., 2002), (Rodriguez et al., 2002), (Tiwari and Hayward, 2002), (Urushitani et al., 2002), (Elam et al., 2003), (Chung et al., 2003), (DiDonato et al., 2003), (Strange et al., 2003), (Rakhit et al., 2004), (Furukawa et al., 2004), (Zhang et al., 2004), (Hough et al., 2004), (Tiwari et al., 2005), (Antonyuk et al., 2005) and (Lindberg et al., 2005).Whereas these modifications alter solubility and promote aggregation *in vitro*, it is not known if the conformational alteration conferred by the mutation is sufficient to cause proteotoxicity *in vivo* or if other post-translational modifications contribute. *In vivo*, the major conformational changes of SOD1 in affected tissues reported are oxidation, protein nitration and 4-hydroxy-2-nonenal (HNE) modifications of amino acids (Pedersen et al., 1998), (Poon et al., 2005), (Perluigi et al., 2005) and (Casoni et al., 2005) and disulfide-reduced SOD1 has been observed in the CNS of mutant SOD1 transgenic mice (Jonsson et al., 2004).

1.3 Protein Quality Control Systems

Many neurodegenerative disorders, including ALS, have in common the sequestration of aberrant proteins into inclusions leading some researchers to name these disorders as "proteinopathies". The identity of the constituent proteins and morphology of the inclusions are pathognomonic of the disease. Proteins within these inclusions often are ubiquitinated, and protein chaperones and proteasomal subunits also may be sequestered (Watanabe et al., 2001), (Sherman and Goldberg, 2001), (Ciechanover and Brundin, 2003) and (Strong et al., 2005). Most investigations of the role of the ubiquitin-proteasome pathway in ALS have been conducted using experimental models of the most common familial form of ALS due to dominantly inherited mutations Cu/Zn-superoxide dismutase (SOD1) (Rosen et al., 1993). Inclusions containing mutant SOD1 are found in motor neurons and sometimes surrounding astrocytes of fALS patients (Shibata et al., 1996a) and (Kato et al., 2000) and transgenic

rodent models (Bruijn et al., 1997) and (Aoki et al., 2005), as well as in primary cultured neurons expressing several different SOD1 mutants (Durham et al., 1997).

The ubiquitin-proteasome system performs many homeostatic functions within cells including nascent protein folding, transport and degradation of proteins, signal transduction, cell growth and differentiation, and control of apoptotic pathways. The proteasome is also the major route for catabolizing misfolded proteins, preventing them from aggregating into insoluble complexes. Protein chaperones facilitate the restoration of functional protein conformation or target misfolded proteins for degradation. These pathways act in concert in ridding cells of unwanted proteins.

Mutant proteins, including SOD-1, misfold acquiring a "toxic gain of function", which in turn leads to exposed hydrophobic patches overloading two of the main cellular defense or "quality control" mechanisms for aberrant proteins, namely the protein chaperones and proteasomes. The main cytosolic protein chaperones, Hsp70 and Hsp90 attempt to refold the misfolded proteins. Proteins in this intermediate misfolded state that cannot be properly folded by chaperones are selectively degraded after being ubiquitinated by the 26S proteasomes (see Figure 1). They can also be degraded independently of ubiquitin and ATP via the 20S proteasome. In animal models, human neurological disorders and normal aging there is extensive evidence illustrating that dysfunction of both chaperone and ubiquitin-proteasome systems could lead to neuronal failure and ultimately cell death (Carrard et al., 2002; Hartl and Hayer-Hartl, 2002; Ross and Pickart, 2004; Ciechanover and Schwartz, 2004).

1.3.1 The role of the ubiquitin-proteasome system in protein turnover

Proteasomes are barrel-shaped protein complexes in the cytoplasm and nucleus responsible for degrading most non-vesicular proteins including short-lived regulatory

molecules, damaged (oxidized, glycated, nitrated, etc.), incompletely translated, and mutant proteins in cells (Baumeister et al., 1998) and (Sherman and Goldberg, 2001). The ubiquitin-proteasome system works in partnership with molecular chaperones, highly conserved families of proteins important for protein folding and transport. Various forms of cellular stress can promote conformational changes that can alter the function of a protein and promote aggregation with like or other proteins (Muchowski, 2002). Misfolding and aggregation are facilitated by molecular crowding in the intracellular milieu (Young et al., 2004). Chaperones aid partially folded or unfolded polypeptides to revert to their functional conformation, preventing their interaction with inappropriate partners and aggregation (Hohfeld et al., 2001). They are also involved in various cellular processes including nascent folding, transport and degradation of proteins, as well as signal transduction, cell growth and differentiation, and preventing apoptosis (Jolly and Morimoto, 2000; Nollen and Morimoto, 2002). The most studied chaperones are the constitutively expressed heat shock cognate proteins (HSCs) as well as stress-inducible heat shock proteins (HSPs) [e,g., Hsp70(68,72,73), 40, 60, 90 and 110; the small HSPs: heme oxygenase, ubiquitin, Hsp25/27, αB-crystallin; the glucose regulated proteins: Grp75,78,94] (Parsell and Lindquist, 1994). They protect cells by refolding or targeting abnormal proteins to the proteasome for degradation. HSPs are induced in response to a variety of stressful conditions (hyperthermia, oxidative stress, ischemia, calcium ionophore, trauma, exposure to toxic chemicals, mutant proteins, etc), a process termed the heat shock response. Cells that can mount a robust heat shock response are better positioned to survive the initial and subsequent insults. Figure 1 summarizes the major pathways by which HSPs and proteasomes regulate protein degradation in cells.

1.3.2 Targeting aberrant proteins to the proteasome for degradation

Although all HSPs have in common the ability to recognize and sequester misfolded proteins, HSP70 and HSP40, with associated proteins and ATP, have the capacity to refold proteins to their functional conformation (Fan et al., 2003; Macario and Conway, 2005). If proteins cannot be refolded, they are escorted to the proteasome for degradation. As a general principle, proteins are targeted to the proteasome by polyubiquitination, a process whereby ubiquitinating enzymes catalyze the covalent attachment of a chain of at least four ubiquitin molecules (Ciechanover, 2005). Ubiquitin is adenylated and activated by binding of E1 (ubiquitin activating enzyme) in an ATP-dependent fashion, then transferred to an E2 ubiquitin conjugating enzyme. Then, HECT domain or RING-finger motif-containing E3 ligases mediate linkage of ubiquitin to lysinyl side-chains of the substrates (Hochstrasser, 2006). Whereas only one mammalian E1 gene exists, hundreds of E3 ligases provide a degree of specificity to ubiquitination of thousands of protein substrates (Ardley and Robinson, 2005). Polyubiquitination is facilitated by E4 enzymes, including C-terminus of HSP70-Interacting Protein (CHIP) (Hoppe, 2005). CHIP shuttles misfolded proteins from HSP70 to be degraded by the proteasome, inhibiting HSP70's refolding activity through N-terminal TPR motifs and facilitating ubiquitination of substrate through its U-box (ubiquitin-ligase) domain (Connell et al., 2001). CHIP promotes degradation of several disease-associated proteins including mutant SOD1 (Choi et al., 2004) and (Urushitani et al., 2004).

Linkage of at least four ubiquitins via lysine 48 targets the substrates to the proteaseome; however, monoubiquitination, or lysine 63-linked ubiquitin chains do not target proteasomal degradation, but serve other functions including signaling, endocytosis, vesicular transport, protein sorting and transcriptional regulation (Voges et al., 1999). The process of ubiquitination is reversed by deubiquitinating (DUB) enzymes (Wing, 2003).

1.3.3 Proteasomal Structure

The 26S proteasome is a complex of approximately 2.5 MDa in size comprised of a 20S catalytic core and two regulatory subunits that recognize and unfold substrates (Baumeister et al., 1998). The 20S proteasome is a cylindrical unit consisting of 28 subunits (14 gene products), arranged as four heptameric staggered rings. The two outer rings contain the structural α subunits (α1– α7), while the two inner rings contain two copies of the β-subunits (β1– β7), three of which (β1, β2 and β5) harbour the six active sites (Kloetzel, 2001). β5 cleaves preferentially after hydrophobic residues [chymotrypsin-like activity], β2 after basic residues [trypsin-like activity], and β1 after acidic residues [caspase-like activity, a.k.a. peptidyl-glutamyl peptide hydrolase (PGPH)] as illustrated in Figure 2. Some of the β subunits are transcribed as proforms and need to be cleaved during the assembly process in order to be functional (Gaczynska et al., 1996). The assembly of the mammalian 20S proteasome is not well understood, but appears to be mediated by POMP or protoassemblin and Hsc73 (Kruger et al., 2001) and (Heink et al., 2005). Recently, two chaperones, PAC1 and PAC2, were identified that associate with proteasome precursors and promote proteasomal maturation (Hirano et al., 2005). The 19S is composed of 18 different subunits and is responsible for recognizing ubiquitinated substrates, unfolding them and chaperoning them towards the proteolytic cavity (DeMartino and Slaughter, 1993). In yeast, the molecular chaperone, Hsp90, plays an important role in assembly of 20S and 19S complexes and maintenance of the resulting 26S proteasome (Imai et al., 2003).

In addition to general protein degradation, a key function of proteasomes is to generate peptides for antigen presentation by MHC class I. The cytokines LPS, TNF-α, and/or IFN-γ induce expression of three alternate catalytic subunits, β5i, β2i, β1i and components of a different regulatory subunit, 11S, also known as PA28 (Stohwasser et al., 2000). The inducible subunits replace the constitutive counterparts forming another complex known as the immunoproteasome (Rivett et al., 2001).Various intermediate/hybrid subtypes of

proteasome also have been isolated including 20S core alone (Shringarpure et al., 2001), 20S associated with one 19S and one 11S subunit (Tanahashi et al., 2000), (Kopp et al., 2001) and (Cascio et al., 2002) and 26S proteasomes with different β subunits. Differences in subunit composition correlate with different hydrolytic activity, the initial cleavage site used and the peptide products generated. Although ubiquitination is the major signal for proteasomal degradation, the 20S proteasome is able to break down proteins in the absence of regulatory particles, particularly oxidatively damaged proteins (Grune et al., 1997).

1.3.4 Regulation of proteasome gene transcription

In comparison to regulation of immunoproteasome subunits, the molecular mechanisms regulating constitutive and stress-induced expression of proteasome subunits in mammalian cells are less well understood. In yeast, coordinated regulation of genes encoding components of the proteasomal and ubiquitin systems is achieved through binding of the transcription factor Rpn4 to a common proteasome-associated control element (PACE) (Mannhaupt et al., 1999) and (Xie and Varshavsky, 2001). Rpn4 regulates proteasome gene expression through a negative-feedback loop. The transcription factor is itself a proteasomal substrate, accumulating when proteasome levels are insufficient, and being quickly degraded when activity is restored (Xie and Varshavsky, 2001) and (Fleming et al., 2002). No homolog of Rpn4 or its DNA-binding element has been identified in the mammalian genome. However, indirect antioxidants stimulate transcription of numerous genes encoding 20S, 19S and 11S proteasomal subunits, in addition to antioxidant/detoxifying enzymes, through the antioxidant response element (ARE), an action dependent on the Keap1-Nrf2 signaling pathway (Kwak et al., 2003a) and (Kwak et al., 2003b). The transcription factor Nrf2 is negatively regulated by binding to Keap1 in the cytoplasm (Motohashi and Yamamoto, 2004) and (Nguyen et al., 2004), which also promotes its degradation by the ubiquitin-proteasome

system (UPS). Thus, like Rpn4 in yeast, Nrf2 is maintained at low levels under low stress conditions by proteasomal degradation (Nguyen et al., 2003). Upon exposure to xenobiotic electrophiles generated by xenobiotic or physiological stress, Nrf2 is released from Keap1, translocates to the nucleus and initiates transcription of a variety of stress response genes including phase II detoxifying enzymes and proteasomal subunits. Peroxisome proliferators also increase expression of proteasome genes, but independent of Nrf2 (Anderson et al., 2004), indicating that proteasome levels can be altered through multiple pathways. Recently, Zif268was identified as a repressor of transcription of some proteasomal subunits (James et al., 2006). Although in yeast, the major heat shock transcription factor (Hsf1) influences expression of ubiquitin-proteasome subunits (ubiquitin, an E3 ligase, and a ubiquitin conjugating enzyme) (Hahn et al., 2004), HSF1 does not appear to regulate proteasome genes or activity in mammalian cells (Taylor et al., 2005).

1.3.5 Methods of measuring proteasomal activity

Distinct catalytic activities have been assigned to each of the three active β-subunits and can be measured in cell/tissue homogenates using fluorogenic peptide substrates specifically hydrolyzed by chymotrypsin-like (β5), trypsin-like (β2), and PGPH/caspase-like (β1) activities (Arribas and Castano, 1990), (Keller et al., 2000a), (McNaught and Jenner, 2001) and (McNaught and Jenner, 2001). This technique measures the ability of proteasomes present in the tissue extract to catabolize an additional substrate load (fluorogenic substrates) and can be expressed relative to a housekeeping protein in the sample (total proteasome activity) or to levels of specific proteasome subunits (specific activity). Proteolytic activity of both 20S core alone and 20S with regulatory particles attached (26S) is measured. The relative contribution to total activity will depend upon the abundance in the tissue and the method of tissue preparation; i.e., whether 26S remains intact or 19S/11S detach. Addition of

glycerol and ATP to the buffer preserves the interaction of 20S core with regulatory particles (Elsasser et al., 2005).

To obtain measures of proteasome activity *in vivo*, fluorescent reporter proteins have been constructed. Kopito's group coupled green fluorescent protein (GFP) to a short degron, CL1 [ACKNWFSSLSHFVIHL] (GFP^u), which promotes its polyubiquitination and degradation by the 26S proteasome (Bence et al., 2001). Dantuma's group has constructed two different ubiquitin-tagged GFP substrates. The first construct, the 'N-end rule' substrate, Ub-R-GFP, in which the N-terminal arginine of GFP is recognized by the E3 Ubr1 after cleavage by deubiquitinating (DUB) enzymes. In the second construct, Ub^{G76V}-GFP, an N-terminally linked ubiquitin serves as the acceptor for polyubiquitination, but the G76V substitution prevents de-ubiquitination, thereby promoting efficient degradation (Dantuma et al., 2000) and (Lindsten and Dantuma, 2003a). All of these reporter proteins have short half-lives because of their efficient degradation by the ubiquitin-proteasome pathway and accumulate when proteasome activity is inhibited; thus increase in GFP fluorescence is a measure of reduced ubiquitin-dependent proteasomal activity. Lines of mice carrying the Ub^{G76V}-GFP (Lindsten et al., 2003b) or GFP-CL1 (GFPdgn) (Kumarapeli et al., 2005) transgene have been generated to provide a readout of proteasome function *in vivo*. The ability to monitor proteasome function in individual cells without lysing tissue is more meaningful than having a measure of activity in homogenate. However, this *in situ* method is not completely straightforward. Only ubiquitin-dependent proteolysis is monitored (i.e., 26S activity) and the GFP reporter can itself aggregate and compromise chaperoning capacity if expression levels are too high (Link et al., 2006). The other issue is that rather high level inhibition of specific proteasome activity, as measured by fluorogenic substrates in homogenates, is required to observe substantial increase in GFP reporter fluorescence (Dantuma et al., 2000), (Lindsten et al., 2003b) and (Bennett et al., 2005). If such levels of

inhibition represent a threshold of function that is only compatible with life over the short term, the reporters may only monitor end-stages of pathogenesis. On the other hand, biochemical measures of proteasome activity do not provide sufficient information on functional impairment or cell type differences. A combined approach using both biochemical and *in situ* techniques is more informative.

Native gel electrophoresis is useful to examine the subunit composition of proteasomes. Separation of 20S, 26S, precursors and unassembled subunits can be accomplished by the 2-D Coomassie-Blue native gel method; tissue lysates are run in the first dimension on Blue native gels, then gel strips are placed horizontally over denaturing SDS-polyacrylamide gels for separation of the complexes into subunits in the second dimension (Camacho-Carvajal et al., 2004). The native gel electrophoresis method described by Finley and colleagues is enriched with ATP and glycerol and favours retention of the 26S complex (Elsasser et al., 2005). In gel activity assays using fluorogenic substrates provides a crude determination of the proteolytic activity in the tissue samples (Elsasser et al., 2005). These are powerful techniques to evaluate proteasome composition in small tissue samples. We are the first to use the native gel electrophoresis methods to determine proteasome assembly and composition in neurodegenerative disorders.

1.3.6 Proteasome Impairment in ALS

1.3.6.1 Interaction of mutant SOD1 with UPS components

Inclusions in spinal cord of several mutant SOD1 transgenic mouse models often are labeled by antibodies to members of the ubiquitin-proteasome pathway (Watanabe et al., 2001). There is biochemical evidence showing that mutant SOD1, but not WT is poly-ubiquitinated both in neuronal and non-neuronal cells and is subsequently degraded by the 26S proteasome (Hoffman et al., 1996; Johnston et al., 2000; Urushitani et al., 2002; Niwa et

al., 2002). Several E3 ligases can poly-ubiquitinate mutant SOD1 *in vitro*. The E3 ligase, dorfin interacts with mutant SOD1 and its overexpression decreases mutant SOD1 toxicity (Niwa et al., 2002). Two other E3 ligases, CHIP and NEDL1 also can interact with and ubiquitinate mutant SOD1 (Choi et al., 2004), (Miyazaki et al., 2004; Urushitani et al., 2004). Using microfluorometric imaging techniques, Matsumoto et al showed stable association of transfected β5i (LMP7) proteasomal subunits with mutant SOD1 inclusions in differentiated PC12 cells (Matsumoto et al., 2005).

1.3.6.2 Focal Dysfunction of the UPS in ALS

Collectively, these data show that mutant SOD1, but not WT SOD1 proteins are catabolized by the proteasome and that biophysical species with altered solubility are precursors of larger inclusions. Under most circumstances cells are capable of handling mutant SOD1 proteins sufficiently to prevent them from exerting toxicity and/or being sequestered into inclusions. Even motor neurons develop and function into adulthood. However, under circumstances of increased physiological or environmental stress or compromise of protective mechanisms with aging, the ubiquitin-proteasome pathway may become overloaded and impaired. This hypothesis was tested in these studies and an impairment of the proteasome activity was determined in our published studies (Kabashi et al., 2004), in *in vitro* and *in vivo* models of fALS (Chapter 4). This impairment was associated with decreases in 20S β catalytic subunits (Chapter 5) but no transcriptional defects and complex malformation were observed. A similar impairment of the proteasome was determined in spinal cord tissue from sporadic ALS patients (Chapter 6).

1.3.7 Role of Protein Chaperones in ALS

1.3.7.1 Reduced protein chaperoning activity

Protein chaperones also play major roles in maintaining protein quality control, working in complementary fashion with antioxidant enzymes and proteasomes. The capacity for both proteasomal protein degradation and protein chaperoning are preferentially reduced in lumbar spinal cord of mutant SOD1 transgenic mice, but not in tissues resistant to disease. In two lines of $SOD1^{G93A}$ SOD1 and $SOD1^{G85R}$ transgenic mice, chaperoning capacity was reduced in the lumbar region of the spinal cord prior to the onset of symptoms (Bruening et al., 1999) and (Tummala et al., 2005). Why chaperoning activity is diminished is not known, but logically would involve increased load of misfolded proteins without increased levels of HSPs/HSCs to maintain chaperoning function. The following evidence supports direct interaction: Hsp70 was detected in the insoluble fraction of spinal cord from $SOD1^{G93A}$, but not $SOD1^{WT}$, transgenic mice and in spinal cord cultures prepared from E13 $SOD1^{G93A}$ mice (Shinder et al., 2001). Similarly, the small HSPs Hsp25 and αB-crystallin cofractionated with mutant SOD1 in a transgenic mouse expressing SOD1 with the four copper-coordinating histidines mutated (Wang et al., 2003). This could result from association with SOD1 or other misfolded proteins. Post-translational modification of Hsp70 by HNE has also been reported in these mice and could affect its chaperoning activity or solubility (Perluigi et al., 2005). Also, HNE-modified proteins are potent inhibitors of the proteasome complex (Friguet and Szweda, 1997). Direct association of HSPs with mutant SOD1 is indicated by the presence of Hsc70 in inclusion bodies in spinal cord of G93A and G85R SOD1 transgenic mice (Watanabe et al., 2001) and by transient association of Hsp70 with mutant SOD1 inclusions in transfected cells (Matsumoto et al., 2005). Urushitani et al. reported transient association of Hsp/Hsc70 with SOD1 and CHIP (Urushitani et al., 2004). However, Hsp70 was not detected in inclusion bodies from mutant SOD1 transgenic mice and was rare in ALS patients (Watanabe et al., 2001) and (Batulan et al., 2003).

1.3.7.2 Regulation of HSP gene transcription

Eukaryotic expression of HSPs is mediated by binding and activation of heat shock transcription factors (HSF) to heat shock elements (HSEs) on *HSP* promoters (Morimoto, 1998), (Voellmy, 2004) and (Westerheide and Morimoto, 2005). Environmental and physiological stresses activate transcription of *HSP* genes in mammals largely through Hsf1 (Morimoto, 1998). In the most accepted model, Hsf1 is sequestered in the cytoplasm bound to a multichaperone complex including Hsp90 and Hsp70. Upon stress, misfolded proteins compete for HSPs, releasing Hsf1 to translocate to the nucleus and bind as a trimer to HSE in *HSP* promoters. Subsequent steps are required to activate Hsf1 including phosphoryation of key residues in its regulatory domain, and release of inhibitory chaperone complexes (Morimoto, 1998), (Voellmy, 2004) and (Voellmy, 2005). Redox-dependent activation of HSF1 through oxidation of cysteine residues could also promote trimerization and DNA binding (Ahn and Thiele, 2003).

Another HSF, Hsf2, is an important developmental regulator (Rallu et al., 1997), but might also be involved in the heat shock response in association with Hsf1 (Sistonen et al., 1994). Like many transcription factors, Hsf2 is a proteasomal substrate and accumulates with proteasome inhibition (Mathew et al., 1998). Treatment of cultured motor neurons with proteasome inhibitors causes both nuclear accumulation of Hsf2 and expression of Hsp70 (Batulan et al., 2003). It is not clear to what extent Hsf2 is responsible, but this pathway is potentially an alternative therapeutic target to enhance HSP production.

1.4 Therapeutical Perspectives

As described above, the development of transgenic animal models has increased the understanding of ALS pathophysiology. Various therapies have been proposed, tested and some of the pharmacological treatments proved quite effective in animal models.

Unfortunately, none of these therapies has worked to increase lifespan and/or quality of life in ALS patients. Since ALS is a multifactorial disease, a combination of pharmacological treatments that target various molecular pathways of ALS pathogenesis will be necessary in the future. Several potential candidates to treat ALS are listed below and a more complete analysis including fALS animal model used, age of animal when treatment started, as well as benefit in disease onset and progression of these treatment is presented in Table 2.

Treatment of $SOD1^{G93A}$ transgenic mice at disease onset with catalytic antioxidant manganese porphyrin significantly increased lifespan and improved motor function of these mice (Crow et al., 2005). Also Nrf2 activation by tert-butylhydroquinone (tBHQ) (Lee et al., 2003) decreased $SOD1^{G93A}$ astrocytic apoptosis and preserved motor neurons in co-cultures (Vargas et al., 2006). Nrf2 regulates transcription of phase II anti-oxidant enzymes, but also increases expression of subunits as well as the proteolytic activities of the proteasome (Kwak et al., 2003); therefore this transcription factor might play an important role in increasing degradation of mutant SOD1 and decreasing its toxic gain of function. Several studies suggested that lowering excitotoxicity might be a determining step in motor neuron survival in ALS. However, treatments with riluzole (reduces glutamate toxicity and modulates AMPA receptors) and ceftriaxone (increases levels of the glutamate transporter, EAAT2, in astrocytes, and limits excitotoxic effects of glutamate by taking up the neurotransmiiter into astrocytes) had moderate effect in increasing lifespan of $SOD1^{G93A}$ transgenic mice (Gurney et al., 1996; Rothstein et al., 2005). Also, pharmacological treatments that can reduce mitochondrial damage, such as Cyclosporin A, might be effective in ALS patients (Keep et al., 2001). Several agents that decrease CNS inflammation and/or microglial reactivity were tested in murine models of ALS (see Table 2). Minocycline (inhibitor of microglia activation) and celecoxib (COX2 inhibitor) were found to be promising therapeutics in several mutant SOD1 transgenic mouse models, but do not seem to be effective in phase III ALS clinical

trials. Finally, delivery of IGF1 and VEGF trophic factors selectively to the affected region (lumbar spinal cord) by viral vector injection at disease onset dramatically improved lifespan and motor function in $SOD1^{G93A}$ transgenic mice by keeping motor neurons alive. Combined treatment using several of these agents that act in different pathways of ALS pathogenesis might increase their efficiency in clinical trials. Also, non-pharmacological treatments, such as exercise and high fat diet, which have been found to be efficacious in the ALS murine model might help slow down ALS progression in patients.

Over the last decade various pharmacological treatments that were found to be effective in animal models of fALS went into clinical trials but did not prove successful. Some of the major failures were usage of only one line of mouse to determine pharmacological benefit (refer to Table 2) as well as non-repeatibility of these published results from independent researchers (refer to http://www.als.net and unpublished lecture by Dr. Robert Brown Jr.). New strategies are required to discover novel pharmacological treatment for ALS patients that would include more rigorous animal studies of pharmacological therapies, generation of new animal models of fALS more suitable than mice for high-throughput drug research, and use of transgenic mouse models with low expression of mutant SOD1.

1.4.1 Treating Protein Misfolding

1.4.1.1 Gene Inhibition Therapy in ALS

The toxic gain of function hypothesis would indicate that inhibiting the action of mutant SOD1 in transgenic mice would alleviate ALS pathology. Indeed, studies from two groups demonstrated that retrograde lentiviral delivery of short hairpin RNAs that inhibit mutant SOD1 gene expression through intramuscular injection in $SOD1^{G93A}$ transgenic mice dramatically preserved motor neuron viability and prolonged lifespan (Ralph et al., 2005;

Raoul et al., 2005). Also, antisense oligonucleotides to human SOD1 extend survival in $SOD1^{G93A}$ transgenic rats (Smith et al., 2006). Even though only 2% of ALS patients have SOD1 mutations, gene therapy might help to decrease or boost expression of gene products that are involved in ALS pathogenesis.

1.4.1.2 Up-regulating Protein Chaperones in ALS

Over-expression of Hsp70 in cultured motor neurons significantly decreased mutant SOD1 toxicity (Bruening et al., 1999), although this chaperone alone did not significantly affect disease in transgenic mice (Liu et al., 2005). However, upregulation of multiple chaperones is more likely to have therapeutic benefit. A combination of Hsp70 and Hsp27 were more protective in combination than individually in dorsal root ganglion neurons transduced to express mutant SOD1 (Patel et al., 2005). Arimoclomol, a coinducer of heat shock gene transcription, improved hind limb muscle function, delayed motor neuron death, and led to a 22% increase in lifespan of $SOD1^{G93A}$ transgenic mice (Kieran et al., 2004). In the primary culture model of fALS, co-expression of a constitutively active form of the major heat shock transcription factor, Hsf1, or treatment with geldanamycin (also known to activate Hsf1) dramatically induced expression of Hsp70 and Hsp40 and was highly protective (Batulan et al., 2003) and (Batulan et al., 2006).

1.4.1.3 Targeting Protein Quality Control Systems

Matching levels of protein chaperones and the ubiquitin-proteasome pathway to the substrate demand in times of stress is highly important to maintain homeostasis. Some cells, some tissues and some individuals do that better than others (Franklin et al., 2005). In models of familial ALS, both proteasome and chaperoning function are abnormal in the tissue most vulnerable to the disease process. Whether this is the case in familial and sporadic ALS

patients is under investigation in our laboratory. The broad spectrum capability for cytoprotection makes these pathways attractive as targets for therapy in ALS, and other neurodegenerative diseases. However, HSPs also can be toxic (Nollen and Morimoto, 2002) and the mechanisms governing their regulation at the transcriptional and post-transcriptional levels are highly complex. Similarly, a generalized increase of the proteasome activity in ALS might be detrimental since this pathway has been involved in muscle wasting conditions. Therefore, a better understanding of the specific mechanisms of proteasome impairment as well as biogenesis, formation of the proteasome complex and transcriptional regulation of its subunits is needed. A better understanding of these mechanisms, development of treatments that upregulate these pathways in a coordinated manner, and use of preclinical test models that evaluate effectiveness of therapeutics in models that represent the cell types affected in the disease are encouraged.

Table 1

Animal models of fALS1

Several lines of mutant SOD1 transgenic mice and rats have been generated in the last decade. Motor neuron loss, glial reactivity, ubiquitin inclusion formation in motor neurons and astrocytes has been determined in most of these models. The first line to be generated by Gurney still remains the most popular of these models when mechanisms of pathogenesis as well as pharmaceutical treatments are tested. Mutant SOD1 has also been overexpressed in other organisms, including *Drosophila* and *C.elegans* where it produced a neuronal pathology. Table was modified from (Bendotti and Carri, 2004).

Model	**Transgene Promoter**	**Neurological Phenotype**			**Disease Onset**	**Complete Paralysis**	**Reference**
		MN Loss	Ubb Inclusion	Glial Response			
Mouse SOD1							
$hSOD1^{G93A}$ (high copy G1H)	Genomic (hemi)	+	+	+	91	136	(Chiu et al., 1995)
$hSOD1^{G93A}$ (slow line G20)	Genomic (hemi)	+	+	+	150	180-210	(Gurney et al., 1994)
$hSOD1^{H46R}$	Genomic (hemi)	+	+	+	140	169	Chang-Hong
$hSOD1^{H46R/H48Q}$	Genomic (hemi)	+	N.D	+	120-180	N.D	(Wang et al., 2003)
$hSOD1^{G37R}$	Mouse Prion	+	N.D	+	301	336	(Wong et al., 1995)
$hSOD1^{G37R}$	Genomic (hemi)	+	+	+	210	330	(Wang et al., 2005b)
$hSOD1^{G85R}$	Genomic (hemi)	+	N.D		240	255	(Bruijn et al., 1997)
$mSOD1^{G86R}$	Genomic (hemi)	+	N.D	+	100	107	(Ripps et al., 1995)
$hSOD1^{G126Z}$	Genomic (hemi)	+	+	+	N.D	240	(Wang et al., 2005a)
$hSOD1^{G127X}$	Genomic (hemi)				114	124	(Jonsson et al., 2004)
Rat SOD1							
$hSOD1^{G93A}$	Genomic (hemi)	+	+	+	99	113	(Howland et al., 2002)
$SOD1^{H46R}$	Genomic (hemi)	+	+	+	145	170	(Nagai et al., 2001)

Table 2

List of pharmaceuticals and their success rates in animal models of fALS1

Some of the most effective pharmacological treatments and their effect in the survival of various ALS murine models are presented. Most of these treatments are tested at various stages in ALS patients. The results of some these drug trials can be viewed at http://www.alsa.org/patient/drug.cfm.

Treatment	Disease Mechanism	Delivery Mode	Molecular/ Cellular Target	ALS Murine model	Start of treatment	Lifespan increase	References
Minocycline	Neuro-inflammation	Diet	Microglial Inhibition	G37R SOD	36 out of 52 weeks	21 days	(Kriz, Gowing, and Julien 429-36)
Celecoxib	Neuro-inflammation	Diet	Cox2 inhibitor	G93A SOD high copy	4 out of 18 weeks	28 days	(Drachman et al. 771-78)
Arimoclomol	Protein Aggregation	IP injection	Hsp co-inducer	G93A SOD high copy	10 out of 18 weeks	23 days	(Kieran et al. 402-05)
IGF-1	Neuronal Apoptosis	Intramuscular adenovirus injection	Motor neuron trophic support	G93A SOD high copy	13 out of 18 weeks	37 days	(Kaspar et al. 839-42)
VEGF	Neuronal Apoptosis	Lentiviral vector injection	Motor neuron trophic support	G93A SOD high copy	13 out of 18 weeks	19 days	(Azzouz et al. 413-17)
Cyclosporin A	Mitochondrial damage	Intrathecal infusion	Mito permeability pore inhibitor	G93A SOD high copy	Hindlimb weakness	12 days	(Keep et al. 327-31)
Manganese porphyrin	Oxidative damage	IP injection	Peroxynitrite decomposer	G93A SOD high copy	12 out of weeks	24 days	(Crow et al. 258-65)
Riluzole	Excitoxicity	Diet	Inhibition of glutamate release	G93A SOD high copy	7 out of 19 weeks	10–15 days	(Gurney et al. 147-57)
Ceftriaxone	Excitoxicity	IP injection	Inactivation of GLT-1	G93A SOD high copy	12 out of 18 weeks	10 days	(Rothstein et al. 73-77)
Oxidized galectin-1	Neurofilament accumulation	Intramusclular injection	Axonal regeneration	H46R SOD	10 out of 23 weeks	12 days	(Chang-Hong et al. 203-11)

Figure 1

The role of protein chaperones and the ubiquitin–proteasome system (UPS) in maintaining protein quality control

During polypeptide synthesis, HSPs act as chaperones to prevent inappropriate protein–protein interactions and assist nascent proteins in taking on and maintaining their functional conformation. Translational errors, genetic mutation or post-translational modifications promote misfolding of proteins, which can expose hydrophobic domains and lead to inappropriate interactions with like or other proteins. These protein aggregates have reduced solubility and tend to come together as insoluble inclusions. HSPs prevent aggregation by recognizing hydrophobic domains of misfolded proteins and sequestering them to be refolded (by Hsp70, Hsp40 and other co-chaperones in an ATP-dependent process) or targeted to the proteasome for degradation. Proteasomes also degrade abnormal proteins from the endoplasmic reticulum, and as such, ER stress also increases the substrate load of cytosolic HSPs and proteasomes. Neurotransmission also depends upon proteasome function. Not only is the UPS involved in turnover of glutamate receptors at the cell membrane, but glutamatergic neurotransmission promotes aggregation of mutant protein in a calcium-dependent manner. Cells that maintain a balance between the levels of HSPs and UPS components and the substrate load will be better positioned to defend themselves. HSP gene transcription is regulated by a negative feedback system whereby the major transcription factor controlling heat shock gene transcription (Hsf1) is maintained in an inactive state by binding to a Hsp90 chaperone complex. When misfolded proteins compete for these chaperones, Hsf1 can translocate to the nucleus and binds as a trimer to heat shock elements (HSE) of heat shock genes. DNA-binding is not sufficient to initiate transcription. Rather subsequent steps are required to activate Hsf1 including phosphorylation and possibly dissociation of inhibitory proteins. Neurons, motor neurons in particular, have a high

threshold for activation of Hsf1, which can compromise their response to disease-related stresses. The major pathway identified in upregulation of proteasomal gene transcription is through antioxidant response elements (ARE) involving the Nrf2–Keap1 pathway which also controls transcription of phase II antioxidant/detoxification enzymes. Nrf2 is sequestered in the cytoplasm by binding to Keap1, which also promotes its degradation by the UPS. Oxidative stress signals dissociation of the Nrf2/Keap1 complex, permitting translocation of Nrf2 to the nucleus and initiation of gene transcription. (For references see text).

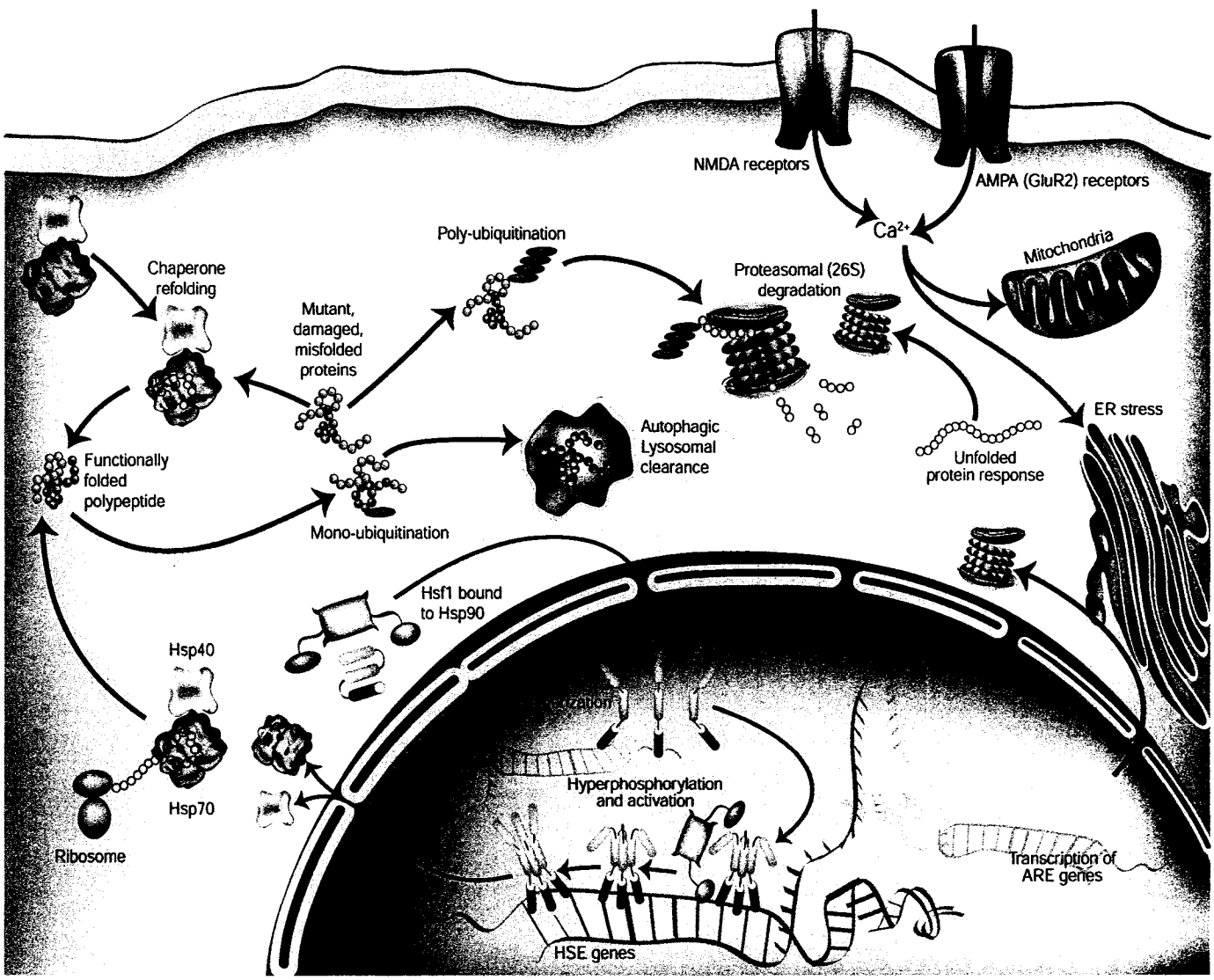
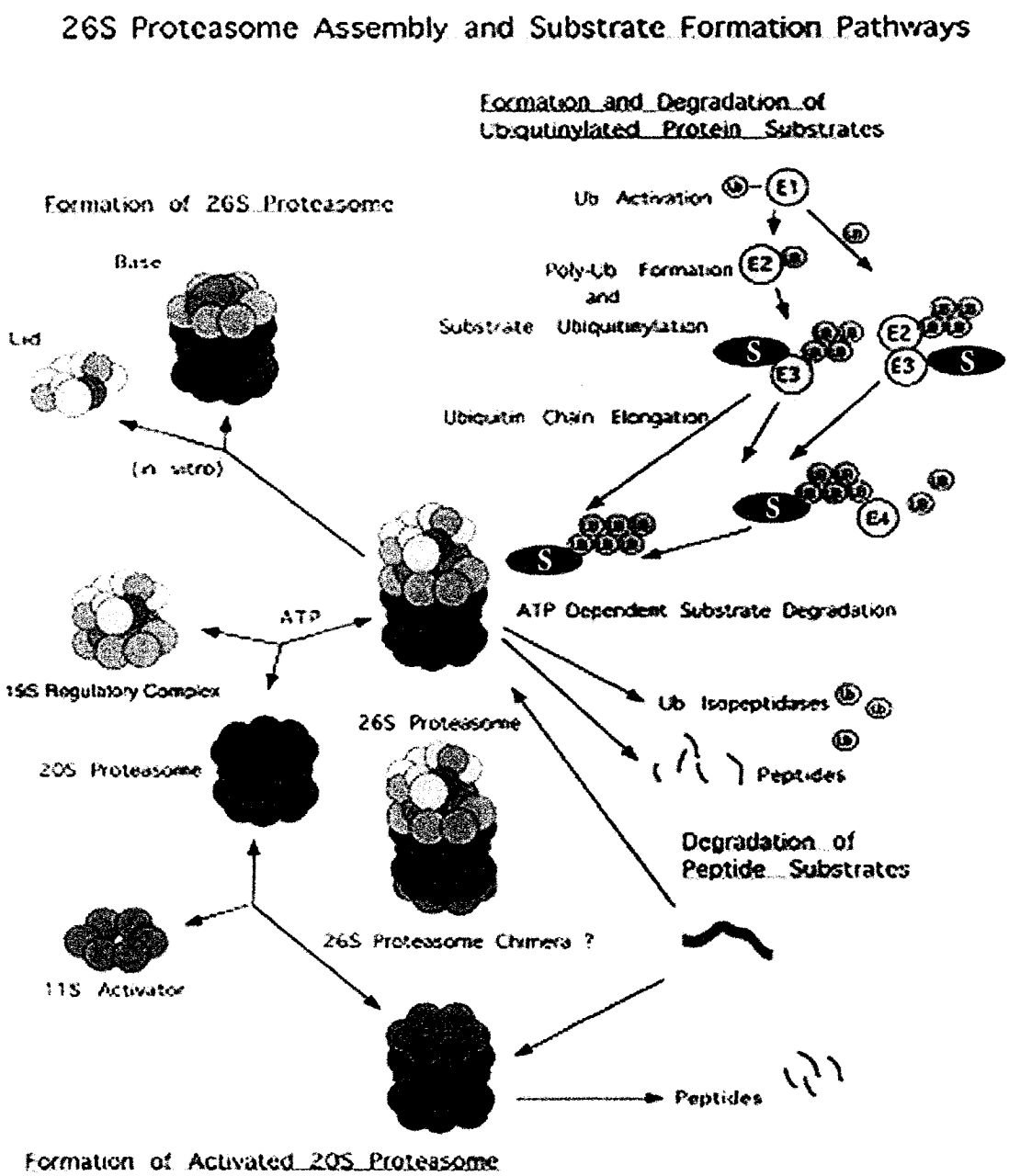

NMDA receptors
AMPA (GluR2) receptors
Ca2+
Mitochondria
Poly-ubiquitination
Proteasomal (26S) degradation
Chaperone refolding
Mutant, damaged, misfolded proteins
ER stress
Autophagic Lysosomal clearance
Functionally folded polypeptide
Unfolded protein response
Mono-ubiquitination
Hsf1 bound to Hsp90
Hsp40
Hyperphosphorylation and activation
Hsp70
Ribosome
Transcription of ARE genes
HSE genes

Figure 1

Figure 2

Ubiquitination process, the structure of the proteasome and its proteolytic activities

Ubiquitin is activated by the ubiquitin-activating enzyme, E1 and transferred to a ubiquitin-conjugating enzyme, E2. HECT domain or RING-finger motif-containing E3 ligases mediate linkage of ubiquitin to lysinyl side-chains of the substrates. Successive conjugation of ubiquitin moieties to one another generates a chain of polyubiquitin bound to the target protein substrate which serves as the binding and degradation signal for the downstream 26S proteasome. The substrate is degraded to short peptides, and DUBs release free and reusable ubiquitin. The proteasome is a large, multicatalytic protease that degrades polyubiquitinated proteins to small peptides composed of the 20S core particle that carries the proteolytic activities, and regulatory particle, 19S and/or 11S. The 20S is a barrel-shaped structure composed of four stacked heptameric rings, two identical outer rings composed of seven α subunits, and two identical inner rings composed of seven β subunits, three of which (β1, β2 and β5) harbour the six active sites. β5 cleaves preferentially after hydrophobic residues [chymotrypsin-like activity], β2 after basic residues [trypsin-like activity], and β1 after acidic residues [caspase-like activity, a.k.a. peptidyl-glutamyl peptide hydrolase (PGPH)]. The 19S is composed of 18 different subunits and is responsible for recognizing ubiquitinated substrates, unfolding them, chaperoning them towards the proteolytic cavity and opening the orifice in the α ring of 20S. For complete references see Section 1.2.6.2 and 1.2.6.3.



Modified from Ferrell et al. 2000

Figure 2

Chapter 2 Rationale and Specific Aims

2.1 Rationale

Treatment with proteasomal inhibitors in cells expressing mutant SOD1 promotes insolubility and accumulation of the mutant protein, as well as formation of microscopically visible inclusions. There is also biochemical evidence that mutant SOD1, but not $SOD1^{WT}$ proteins are polyubiquitinated and rapidly degraded by the proteasomal machinery. Therefore, it would be logical that the presence of inclusions in cells affected in ALS would indicate compromise of proteasomal function; i.e., overload of the proteasome or reduced efficiency in degrading mutant SOD1 and/or other substrates resulting in their accumulation and increasing the opportunity for aberrant protein interactions and aggregation into insoluble complexes. Moreover, impaired function of the proteasome, whether due to substrate overload or altered composition and/or assembly of proteasomal complexes (20S, 26S, immunoproteasome) could disrupt the proteolysis of other important cellular proteins that are crucial for cellular homeostasis including transcriptions factors, kinases, and caspases. This study set out to examine the role of the proteasome in FALS by testing the following hypothesis:

2.2 Specific Aims

General Hypothesis: Expression of mutant SOD1 and other misfolded proteins abrogates proteasomal function and contributes to fALS pathogenesis.

Specific Aim 1: To determine if levels, localization and proteolytic activities of the proteasome are impaired during disease pathogenesis in various affected and unaffected tissues from a murine model of fALS

Specific Aim 2: To determine mechanisms that lead to *in vivo* proteasome impairment and consequences of proteasome dyfunction in a murine model of fALS

Specific Aim 3: To determine if levels, localization and activities of the proteasome complex are affected in autopsy tissue from sporadic ALS patients

2.3 Choice of *In Vivo* Experimental Model

In order to determine the role of the ubiquitin-proteasome pathway in the pathogenesis of ALS, we have used the transgenic mouse line B6SJL-TgN(SOD1^{G93A})1Gur overexpressing a high copy number of the gene encoding human SOD1 with the ALS-causing mutation, G93A, and the line overexpressing similar levels of human SOD1^{WT} as control, both expressed from the SOD1 promoter. This model offers several advantages. Since its generation over 10 years ago, hundreds of reports have been published using this model, characterizing the histological, biochemical, electrophysiological and behavioral phenotype. The highly reproducible time course of these changes permitted us to analyze proteasomal activity and composition at well-defined stages of pathogenesis, to assess function in both vulnerable regions of the nervous system and in organs more resistant to mutant SOD1 toxicity, and to determine if any dysfunction was an early event or occurred later in the disease as a consequence of neuronal damage and/or death. These data are presented in Chapter 4.

Several mechanisms could lead to proteasome dysfunction, including direct inhibition of the proteasome by mutant SOD1 or other substrates, changes in subunit gene and/or protein expression, and/or impairment of the assembly of the proteasome complex. These results are presented in detail in Chapter 5.

Finally, since only a small percentage of ALS is genetically inherited it is important to determine the relevance of the protein quality control systems to the disease in general,

particularly in sporadic ALS. The levels of proteasomal subunits as well as its proteolytic activities were assessed in autopsy tissue from ALS patients and age-matched non-neurological cases. The results from these experiments are presented in Chapter 6.

Chapter 3 Materials and Methods

3.1 Transgenic Mice

All experiments were approved by the McGill University Animal Care Committee and followed the guidelines of the Canadian Council on Animal Care. Two lines of mice were used in this study. B6SJL-TgN(SOD1^{G93A})1Gur, transgenic for human SOD1 with the ALS-associated mutation, G93A, and B6SJL-TgN(SOD1^{WT})2Gur, transgenic for human SOD1^{WT}, which express similar levels of human SOD1 protein (Dal Canto and Gurney, 1995). These lines of mice were obtained from Jackson Laboratory (Bar Harbor, ME, USA) and maintained in our animal facility (Montreal Neurological Institute). Mice hemizygous for the transgene were obtained by breeding hemizygous males with non-transgenic B6SJL females. Mice were genotyped for human SOD1 as previously described (Shinder et al., 2001) and litters including at least three transgenic and three non-transgenic littermates were used at P30, P45, P75 and at symptomatic stage (approximately P120). SOD1^{G93A} transgenic mice were designated 'symptomatic' by positive hindlimb extensor reflex. Mice were euthanized by deep anesthesia with intraperitoneal injection of a mixture of 180 µl ketamine and 20 µl xylazine. Tissues from spinal cord and liver were collected, weighed, and placed immediately on ice. The lumbar enlargement ($L_{I\text{-}VI}$) and thoracic ($T_{I\text{-}XIII}$) segments of the spinal cord as seen in Figure 3 were processed separately.

3.2 Human Autopsy Tissue

Post-mortem human tissue was obtained from Dr. Michael Strong, University of Western Ontario. Tissue was frozen at -80°C immediately after autopsy. Sufficient spinal cord tissue (100 mg - 1 g) for biochemical analysis from the cervical region and the thoracic region from 5 ALS patients and 4 controls was shipped in dry ice. Also cerebellar tissue from these

ALS patients and controls was shipped to serve as an internal control for tissue that is not substantially affected in the disease. ALS patients and control individuals were matched as closely as possible for age, sex, and postmortem interval to autopsy account; these data are presented in Table 3. Cross-sections of spinal cord were cut into ventral and dorsal sections and protein levels and proteolytic activities of the proteasome were determined in individual sections of the spinal cord. All handling of human tissue was carried out using standard universal precautions according to biosafety guidelines and with an approved McGill University Biohazard Certificate. Spinal cord sections from adjacent areas of spinal cord of ALS patients as well as controls used for the biochemical analysis reported in Chapter 6 were fixed in 10% formalin overnight and the processing, embedding and sectioning of paraffin blocks was performed according to standard protocols in the Pathology Laboratory at the Robarts Research Institute (London, ON). Spinal cord sections were cut into 6 μm thick and slide mounted in order to determine motor neuron pathology as well as glial reactivity in these tissues.

3.3 NIH-3T3 cells

Clones of NIH 3T3 cell lines stably expressing human SOD-1^{WT} or with the disease-related mutations, SOD-1^{G93A} or SOD-1^{G41S},were previously established (Bruening). SOD1 expression was determined by Western blotting and immunocytochemical analysis with appropriate human SOD1 antibodies (see Antibody section). Cells were cultured in minimal essential medium enriched with 5 g/l glucose supplemented with 10% fetal calf serum and 250 μg/ml of Geneticin (Invitrogen Corp., Grand Island, NY) for mutant lines and 700 μg/ml for wild type lines to maintain selection. Cells were used for experimentation at 80-100% confluency. Cells were subcultured by trypsin-dissociation.

3.4 Tissue Preparation

For proteasome activity assay and SDS/PAGE gel electrophoresis, tissue was suspended in Tris/EDTA solution [20 mM Tris/ 2 mM EDTA, pH 7.5] at a concentration of 0.1 g/ml (spinal cord) or 0.4 g/ml (liver), homogenized for 2 min using a Dinamix homogenizer (Fisher Scientific Co., Nepean, ON), aliquoted, flash frozen in liquid nitrogen and stored at -80 C in 100 µl aliquots. For assay, samples were thawed on ice. Thirty minutes following addition of 5 µg DNase and 400 µl TRIS/EDTA, samples were sonicated for three 10 sec cycles at 50% duty (Vibrocell, Sonics and Materials Inc., Danbury, CT), and centrifuged at 15400 x g for 10 min. Supernatants were collected for determination of protein concentration (DC protein assays, BioRad Laboratories, Hercules, CA), proteasome activity assays, and Western blotting. Pellets were resuspended to original volume in Tris/EDTA by trituration and vortexing and were washed three times with 0.1% Triton X in Tris/EDTA solution and in 0.1% SDS in Tris/EDTA solution. In the final step, pellets were resuspended in saturated 10 M urea in 1/30 of the initial volume of Tris/EDTA used to resuspend the tissue to determine and compare the levels of insoluble to soluble proteins. Tissue extracts were used only once after thawing, i.e., not refrozen. Preliminary Western blotting and proteasome activity assays indicated that approximately 90% of proteasome α-subunits and the three proteasomal activities were in the supernatant of symptomatic mutant SOD-1^{G93A} and their age-matched LM spinal cord tissue. Therefore, supernatants were used in subsequent experiments and taken to be representative of total proteasome.

A different buffer (50 mM Tris, pH 7.4, 10% glycerol, 5mM ATP, 5 mM $MgCl_2$) was used to homogenize tissue for native gel including ATP and glycerol in order to preserve the integrity of 26S proteasomes. Preliminary native gel experiments determined that flash-freezing the samples in liquid nitrogen did not affect the composition and assembly of the proteasome, hence tissue was thawed in ice and loaded in gels.

For immunohistochemistry and laser capture for real-time (rt) PCR analysis of motor neuronal mRNA, cervical, thoracic and lumbar segments of the spinal cord were embedded in Tissue-Tek frozen tissue embedding medium (OCT) (VWR Torrance, CA) on liquid nitrogen cooled 2-methylbutane (American Chemicals Ltd., Montreal, QC). The blocks were stored at -80°C. Frozen sections were cut using a Microm Model HM 500 M Cryostat (Microm International, Walldorf, Germany) as described below.

3.5 Proteasome activity

Assays were based on generation of the fluorescent species, amino methyl coumarin (AMC), from peptide substrates specific for individual 20S/26S proteasome hydrolytic activities as described previously (Arribas and Castano, 1990; Rock et al., 1994; Keller et al., 2000a). Each assay was conducted in the absence and presence of proteasomal inhibitor [either 20 μM MG132 or 50 μM lactacystin, which gave indistinguishable results]. Assays were conducted in 96 well opaque plates using 50 μl of tissue sample or cell line extracts and 50 μl of 2X Proteasome Buffer [4 mM ATP; 10 mM $MgCl_2$, 8 mM dithiothreitol (DTT)]. To avoid experimental activation of 20S proteasomes, no SDS was included in any assay buffer. All samples were assayed in quadruplicate using 10 μg of total protein with and without proteasomal inhibitor. Chymotrypsin-like, trypsin-like, and caspase-like activities were measured by adding 2.5 μl of 1 mM Suc-LLVY-AMC peptide, Boc-LRR-AMC or Z-LLE-AMC, respectively (Peptide International Inc., Louisville, KY). Fluorescence of generated AMC was measured at intervals of 30 min for 3 hrs using a FX600 multiplate reading fluorometer (Bio-Tek Instruments, Inc., Winooski, VT). All assays were linear in this range. As a standard, free AMC was added to separate wells at the same concentration as in fluorogenic peptides. To convert AMC generated from peptide substrates to nmol of AMC/min/mg protein, values in fluorescence units/min/mg protein were divided by

fluorescence units of free AMC measured at 150 min. To obtain total proteasome activity, this value was normalized to actin levels quantified on Western blots using enhanced chemifluorescence. A measure of specific proteasomal activity was determined by subsequent normalization to the level of 20S proteasome core α subunits (see Western blotting and Antibodies below). Removal and initial processing of tissues from an entire mouse litter (n = 4 ± 1 for transgenic and n = 4 ± 1 for LM) were carried out simultaneously. Similarly, all samples of lumbar and thoracic spinal cord from an entire litter were processed simultaneously in protein assays, proteasomal activity assays, and Western blots. Liver tissues from SOD-1^{WT} and SOD-1^{G93A} transgenics and their respective LM for a given age group were processed and assayed simultaneously. Untransfected NIH 3T3 cell lines, and three clones stably expressing human SOD-1^{WT}, SOD-1^{G93A} and SOD-1^{G41S} were assayed simultaneously.

3.6 Gel electrophoresis

3.6.1 SDS–PAGE/Western blotting

SDS/PAGE gels with a separating dimension of 15% acrylamide and a stacking dimension of 4% were used in these experiments. Ten μg of total soluble protein was loaded in each lane. Gels were run until the blue colour of the Laemli loading buffer ran out of the gel. Gels were transferred to nitrocellulose membranes overnight at a constant voltage of 40 V at 4°C for low-weight proteins (10-30 kDa) and at 120 V for 3 hours for higher-weight proteins (30-100 kDa).

3.6.2 2D-Coomassie/Blue Native Gel Electrophoresis

The method of Blue native gel electrophoresis allows for two dimensional high-resolution separation and identification of multi-protein complexes. In the first dimension,

multi-protein complexes are separated under native conditions. Each lane of the gel is cut and is run in a second dimension in the presence of SDS. This dimension allows further resolution of the components and subunits that compose multi-protein complexes according to their molecular weight.

The method in Camacho-Carvajal et al. (2004) was used with few modifications to determine *in vivo* proteasome composition and assembly from spinal cord tissue extracts. Briefly, a continuous native acrylamide gradient gel (4-15%) was poured with no stacking gel. Tissue lysates containing 50-100 μg protein were loaded in each lane and electrophoresed at 100 V. Molecular weight markers were run on each gel (Thyroglobullin: 669 kDa; Ferritin: 440 kDa; Catalase: 232 kDa and lower) (Amersham Biosciences, UK). When the blue front had run approximately 2/3 of the way through the gel, the cathode buffer was replaced with buffer lacking Coomassie blue and the power supply was interrupted when the front reached the bottom of the gel. Individual lanes were excised using a sharp scalpel blade and equilibrated in 2X SDS Laemli Buffer with 5% β-mercaptoethanol for 20 minutes, then washed 5 times with the same buffer without β-mercaptoethanol. An individual strip was placed on top of a second-dimension SDS-PAGE gradient gel (4-15%) of the same thickness and run as previously described.

3.6.3 1D-Native Gel Electrophoresis

The methodology described in Elsasser et al. (2005) was followed with the few modifications listed below. The homogenization buffer was 50mM Tris, pH 7.4, 10% glycerol, w/ ATP (5 mM) and $MgCl_2$ (5 mM). Samples containing 50-80 mg of protein were run on gels consisting of three layers of acrylamide (3, 4 and 5%) including rhinohide acrylamide gel strengthener (Molecular Probes) that preserves the integrity of low percentage acrylamide gels (Vernace et al. 2006). The gels were run at 110 V for 3 hours, then

transferred onto nitrocellulose membranes at 45 V overnight at 4°C. The resolving buffer used for making and running the gels was 90 mM Tris, 90 mM boric acid, pH 8.3 w/ ATP (1 mM), $MgCl_2$ (5 mM) and EDTA (0.5 mM). Preliminary native gel experiments determined that flash-freezing the samples did not affect the composition and assembly of the proteasome, as long as ATP and glycerol are added to the samples prior to loading in the gel.

3.6.4 Western Blotting and Analysis

All gels (including the native gels) were transferred onto nitrocellulose paper at 4°C overnight at 45 V or for 3 hours at 120 V depending on the molecular weight of proteins being probed. Membranes were blocked in 3% bovine serum albumin in TBS with 0.1% Tween20, probed with primary antibodies and secondary antibodies corresponding to the species the primary antibodies were raised. Western blots were exposed to Western Lightning chemiluminescence reagents (PerkinElmer, Boston, MA) or SuperSignal West Femto substrates (Pierce, USA) and exposed on Kodak Film. The films were scanned as high-resolution images and the bands corresponding to the appropriate molecular weight of the protein were quantified using Scion Image (NIH) software. Three to four mice were used for each of the following groups: $SOD1^{G93A}$ mice and their non-transgenic littermates (GLm), $SOD1^{WT}$ mice and their non-transgenic littermates (WLm). Every Western blot was repeated twice for each of the proteasomal subunits and reprobed for actin which was used as loading control. Protein levels for each experimental condition (groups of mice) were normalized to actin levels and were quantified as means ± standard error of means (SEM). A two-tailed distribution, two-sample equal variance t-test was used to assess significant difference between the actin-normalized ratios of $SOD1^{G93A}$ mice and each of the other three experimental groups.

3.7 Immunohistochemistry

Twenty μm cross-sections of murine spinal cord were fixed in 3% paraformaldehyde in phosphate buffered saline (PBS), pH 7.3 for 30 min, permeabilized in 0.5% Nonidet-P40/PBS for 5 min, and further fixed in 3% paraformaldehyde for 2 min. Sections were blocked in 3% bovine serum albumin (BSA) for 3 hr at room temperature, then in AffiniPure F_{AB} fragment anti-mouse IgG H+L (Jackson ImmunoResearch Laboratories Inc.,West Grove, PA) for 30 min. Primary antibody in blocking solution was applied overnight (15-17 hr at 4°C) and secondary antibodies were applied for 1 hr at room temperature. Antibody labeling was visualized using Vectastain ABC (Vector Laboratories Inc., Burlingame, CA) with 3,3'-diaminobenzidine tetrahydrochloride dihydrate (ICN Biomedicals Inc., Irvine, CA) as substrate.

3.8 Real-Time PCR

3.8.1 Laser Capture Microdissection (LCM)

Ten μm cryostat sections of mouse lumbar or cervical spinal cord were placed on LCM microslides (Arcturus, Mountain View, CA) and immediately placed in a slide box on dry ice. Unless immediately processed, the slides were stored at -80°C. For LCM, selected slides were thawed for 30s, hydrated by sequential immersion into 75%, 50% ethanol and DEPC treated water for 30s, stained with 1% cresyl violet acetate for 1min. Slides were then dehydrated by sequential immersion into 50%, 75%, 95% and 100% ethanol. Finally the slides were cleared in xylene twice for 3 min each and air-dried. Using a PixCell II Laser Capture Microdissector (Arcturus, Mountain View, CA) according to manufacturer instructions, motor neurons were captured onto CapSure HS LCM caps using the laser spot size of 7.5 μm diameter. Motor neurons were identified as the large cresyl violet stained neurons in the ventral horn of the spinal cord section.

3.8.2 RNA Isolation and Reverse Transcription

Microdissected motor neurons were immediately processed using the PicoPure RNA isolation Kit (Arcturus, Mountain View, CA). Approximately 50 microdissected cells were collected onto each LCM cap. 20 µl of extraction buffer were placed into the LCM cap/ExtracSure/0.5 ml microcentrifuge tube assembly and incubated for 30 min at 42°C. After incubation, total RNA was extracted according to instructions and treated with DNase. Reverse transcription was performed using iScript cDNA Synthesis kit (Bio-Rad, Hercules, CA). Briefly, 4 µl of 5x iScript Reaction Mix, 1 µl of iScript Reverse Transcriptase and total RNA template were included in a final volume of 20 µl. Reaction conditions were 5 min at 25°C, 30 min at 42°C, and 5 min at 85°C.

3.8.3 SYBR Green Real-Time PCR

All rt-PCR reactions were carried out in 96-well 0.2 ml PCR plates sealed with iCycler Optical Sealing tapes (Bio-Rad, Hercules, CA, USA). PCR was performed using cDNA from laser captured motor neurons and the primers for mouse 20S β3 (PSMB3 – PCR product 289 bp), 20S β5 (PSMB5 – PCR product 202 bp), 20S β5i (LMP7 – PCR product 82 bp), 20S α7 (PSMA3 – PCR product 168 bp), and GAPDH (see Table 4). The PCR reactions contained: 1X SYBR Green iCycler iQ mixture (Bio-Rad, Hercules, CA, USA), 0.2 µM of each forward and reverse primer and cDNA preparation in a 25 µl total volume. Amplification and fluorescence detection were performed using iCycler iQ Real-time Detection System (Bio-Rad, Hercules, CA, USA). The PCR amplification profile was: 95°C for 5 min, 45 cycles of denaturation at 95°C for 30 s, annealing at 58°C for 1 min, and extension at 72°C for 30 s, followed by 72°C for 5 min. At the end of the PCR, melting curves were obtained from 46 subsequent temperature increments, by measuring fluorescence every 10 s with +0.5°C/step

increments and beginning at 72°C. The quality of PCR products was determined by melting curves analysis. The fluorescence threshold value (CT) was calculated using iCycler iQ system software and the levels were normalized to values obtained for GAPDH. A nontemplate control (NTC) was run with every assay.

3.8.4 Primer specificity

PCR was performed by cDNA from laser captured motor neurons and whole tissue with specific primers for mouse 20S β3 (PSMB3 – PCR product 289 bp), 20S β5 (PSMB5 – PCR product 202 bp), 20S β5i (LMP7 – PCR product 82 bp), 20S α7 (PSMA3 – PCR product 168 bp), and GAPDH. For the primer sequence refer to Table 4. The results from rt-PCR quantitation were expressed as means ± SEM.

3.9 Antibodies

Primary antibodies used for immunohistochemistry (IH), immunofluorescence microscopy (IF) and Western blotting (WB) were: rabbit anti-GFAP (Z0334 Dako Corp., Mississauga, ON. IH 1 : 1000); rat anti-MAC-1 (a kind gift from Dr. Samuel David. IH 1 : 10); mouse anti-SOD-1 (SD-G6 Sigma-Aldrich Co., St. Louis, MO. IH 1 : 5000); mouse anti-actin (clone C4 ICN WB 1 : 10000); mouse anti-neurofilament 68Kd (NF-L; clone NR-4 ICN; IH 1 : 200); mouse antibody to ubiquitinated (mono and poly) proteins (clone FK2 WB 1 : 2000, IH 1 : 100); and mouse antibody to 20S proteasome subunits α1,2,3,5,6&7 (clone MCP231; WB 1 : 2500, IH 1:50) β3 (clone HC10) (1 : 100 IHC; 1 : 3000 WB); polyclonal antibodies to β5i (LMP7 (1 : 100 IHC; 1 : 2000 WB), 19S5a (1 : 3000 WB), 19S6b (1 : 2000 WB), and 11Sα, all from Biomol (Plymouth Meeting, PA). Monoclonal antibody to Hsp70 (clone SPA810) (1 : 1000 WB), rabbit polyclonal to Hsp40 (SPA400) (1 : 5000 WB), and polyclonal antibody to SOD1 (SOD100) (1 : 3000 WB) were obtained from Stressgen

Biotechnologies (Ann Arbor, Michigan). Monoclonal antibody to actin was purchased from ICN (Irvine, CA) (1 : 10000 WB). Mouse monoclonal antibody (clone 6C5) to GAPDH antibody was a kind gift from Dr. Peter McPherson (1 : 500 IHC).

Secondary antibodies were: Alexafluor-conjugated anti-rabbit, anti-mouse, or anti-rat IgG(H+L) (Molecular Probes Inc., Eugene, OR. 1 : 100) for IF; biotinylated anti-rabbit or anti- mouse IgG(H+L) (Vector; 1 : 100) or anti-rat IgG(H+L) (Jackson; 1 : 100) for IH; anti-rabbit or anti-mouse IgG(H+L) conjugated to horseradish peroxidase (Dako; 1 : 3000) or fluorescein (Amersham; 1 : 1000) for WB.

3.10 Immunoprecipitation

Immunoprecipitation experiments were performed as previously described (Shinder et al., 2001). Briefly, 3 µl of SOD-1 antibody (SOD-100) was added to 400 µg of total protein from soluble tissue extracts and incubated at 4°C overnight on a rotating platform. Immune complexes were captured on 40 µl of protein G-Sepharose, 4 Fast Flow (Amersham Pharmacia Biotech) by incubation on a rotating platform at 4°C for 3 h; the beads were washed with lysis buffer, resuspended in 30 µl of 1× sample buffer, and boiled for 5 min. The beads were pelleted, half of the supernatant was electrophoresed on a 12.5% SDS-polyacrylamide gel, and immunoprecipitated proteins were detected by Western blotting.

3.11 Statistical Analysis

The results from Western blots, proteasome activity assays and rt-PCR quantitation were expressed as means ± SEM. To determine statistical significance, a two-tailed distribution, two-sample equal variance *t*-test was performed using Microsoft Excel and SigmaPlot softwares.

Table 3

Autopsy time, age and sex of sporadic ALS patients and control cases and the tissues obtained

Tissue was obtained from Dr. Michael Strong bank at the Robarts Research Institute in London, ON. Tissue was shipped frozen and processed as described in Chapter 3.

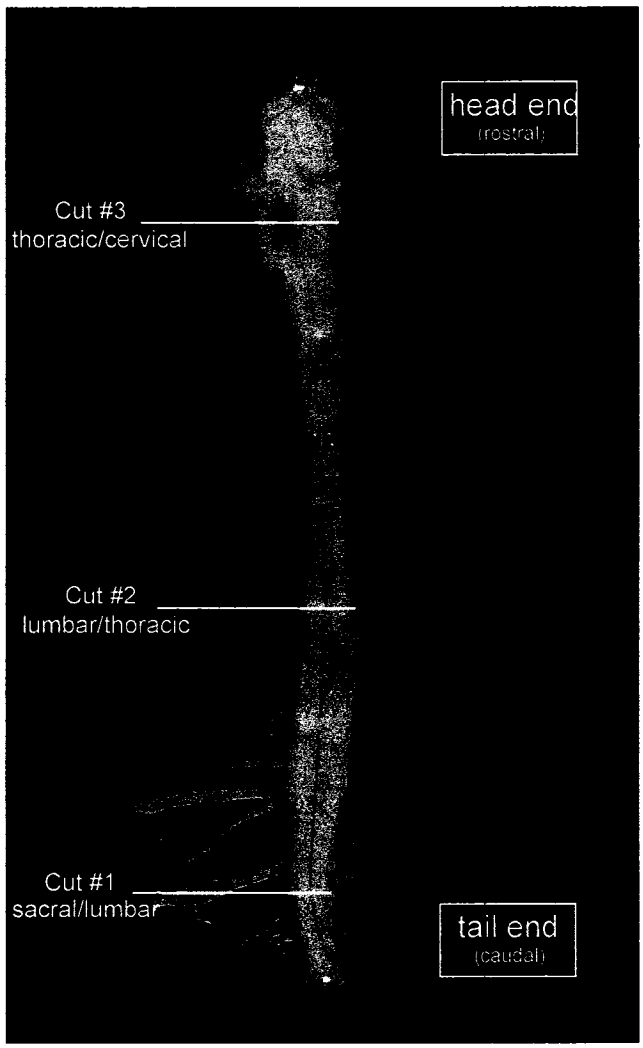
ALS Patient I.D.	**Sex**	**Age**	**Time of autopsy**	**Tissue obtained**
98-A027	M	50	Same day	Thoracic S.C, Cerebellum
00-A183	F	52	N/D	Thoracic S.C, Cerebellum
00-A410	M	72	Same day	Thoracic S.C, Cerebellum
00-A417	F	38	10`10``	Thoracic S.C, Cerebellum
01-A217	M	68	3`30	Thoracic S.C, Cerebellum
Control cases I.D.				
93-A062	M	71	14`30``	Thoracic S.C, Cerebellum
94-A190	F	82	15`45``	Cervical S.C, Cerebellum
96-A146	M	58	15`	Cervical S.C, Cerebellum
99-A071	F	72	19`40``	Thoracic S.C, Cerebellum

TABLE 4

Primer sequence for genes encoding proteasomal subunits used to determine mRNA levels by rt-PCR.

These primers were obtained from the literature in studies that measured mRNA levels of murine proteasomal subunits. The rest of the primers were designed by Yu Hong at the University of Michigan Ann Arbor.

Gene Name	Primer Sequence	Product	Reference
PSMB3 Forward	TTCAGCGTCCTGGTGGTGAT	*289 bp*	
PSMB3 Reverse	ACAGAGCCTGTCATTGCTGG		
PSMB5 Forward	GCTGGCTAACATGGTGTATCAT	*202 bp*	Kwak et al. 2003
PSMB5 Reverse	CACTTTCAGGTCATAGGAGTAGCC		
LMP7 Forward	AGGAGTCGTCAACATGTACCACA	*82 bp*	Allen et al. 2004
LMP7 Reverse	ACTTGTACAGCAGGTCACTGACATC		
PSMA3 Forward	TAGGTCCAACTTCGGCTATAACA	*168 bp*	
PSMA3 Reverse	GATGGGTCGATCATATAGAGCTG		
GAPDH Forward	AGTATGTCGTGGAGTCTACTGGTG	*152 bp*	
GAPDH Reverse	TGAGTTGTCATATTTCTCGTGGTT		



Lumbar and thoracic spinal cord segments in mice Spinal cord was obtained after careful dissection and spinal cords were cleaned from the ventral and dorsal roots and only the spinal cord tissue was used for further experimentation. Tissue was cut in three different places as indicated in the figure. The lumbar and thoracic sections of each mouse were processed separately in our biochemical analysis of the ubiquitin-proteasome pathway.

Figure 3

Chapter 4 Focal Proteasome Impairment Plays a Critical Role in Pathogenesis of a Murine Model of ALS

This chapter reports results of experiments to test Specific Aim 1: To determine if levels, localization and proteolytic activities of the proteasome are impaired during disease pathogenesis in various affected and unaffected tissues from a murine model of ALS. Chymotrypsin-like, trypsin-like and caspase-like activities were measured in homogenates of various tissues from $SOD1^{G93A}$, $SOD1^{WT}$ and nontransgenic littermates from an early, pre-symptomatic stage (P45) through to development of motor dysfunction (symptomatic) and correlated with extent of pathological abnormalities. Specific proteasomal enzyme activity was reduced in lumbar spinal cord of $SOD1^{G93A}$ mice, prior to motor neuron death and reactive gliosis, but affected much later and to a lesser extent in the less vulnerable thoracic region of the spinal cord and unchanged in liver. These data were published in 2004 as Kabashi et al. J. Neurochem. 89:1325-35.

4.1. Choice of Ages to Measure Proteasome Function in Mice Based on Neuropathology

Time points for measuring proteasome activity were based on previous characterization of neuropathological and clinical disease in the B6SJL-TgN($SOD1^{G93A}$)1Gur line of transgenic mice and verification of certain endpoints (glial activation, detergent-insoluble mutant SOD1) in mice of the same line raised in our laboratory.

Clinical disease in the mutant SOD1 transgenic mice begins most obviously with hindlimb involvement and several studies have demonstrated that neuropathological changes developing in the lumbar spinal cord, which contains the motor neurons innervating these limbs. Loss of lumbar and cervical motor neurons reaches statistical significance at approximately P90 (Gurney et al., 1994; Chiu et al., 1995; Chiu et al., 1995). Interestingly, no

significant loss of thoracic or cranial motor neurons occurred, although some cytopathology was noted in symptomatic mice (Chiu et al., 1995).

Earlier, presymptomatic changes include the presence of detergent-insoluble, high molecular weight complexes of SOD1 on Western blots of spinal cord from P30 mice (Johnston et al., 2000) vacuolation of mitochondria in ventral root axons at 37 days of age and motor neuronal perikarya at P45 (Chiu et al., 1995), and up-regulation of ICAM-1 and the immunological markers, IgG and FcγRI, in ventral spinal cord of P40 mice (Alexianu et al., 2001). Mitochondrial abnormalities in lumbar motor neurons first documented as swelling and vacuolation of mitochondria as early as P37 (Chiu et al., 1995). Activation of the unfolded protein response of the ER was demonstrated by up-regulation of two ER-resident chaperones, protein disulfide isomerase (PDI) and endoplasmic reticulum protein 57 (Erp57) as early as post-natal day 60 (Atkin et al., 2006). Cytoplasmic inclusions containing mutant SOD1 were visible in sections of lumbar spinal cord by immunhistochemistry at P112 of $SOD1^{G93A}$ mice (Watanabe et al., 2001) and were isolated from symptomatic mice using a filter-trap assay (Wang et al., 2002).

Experimental Approach and Results

4.1.1 Gliosis at Different Stages of Disease Pathogenesis

Increased numbers of microglia and reactive astrocytes are detected about P75 and increase thereafter (Hall et al., 1998; Alexianu et al., 2001; Batulan et al., 2003). Because death of motor neurons and glial reactivity could potentially alter proteasome activities measured in tissue extracts, the presence of gliosis was evaluated by immunohistochemistry in this study. Markers of reactive astrocytes (GFAP immunoreactivity) (Figure 4A), and of microglia (MAC-1 immunoreactivity) (Figure 4B) were minimal in lumbar spinal cord of P45 $SOD1^{G93A}$ mice, but were evident by 75 days of age and progressively increased until the time

that symptoms appeared (Figure 4), in accordance with previous studies (Hall et al., 1998; Alexianu et al., 2001; Batulan et al., 2003). These pathological markers were not prevalent in the lumbar spinal cord from LM or $SOD1^{WT}$ transgenic mice [B6SJL-TgN(SOD1)2Gur] at any ages examined.

These pathogenic markers defined three stages of disease at which to determine the role of the ubiquitin-proteasome pathway: P45, early pre-symptomatic, prior to motor neuron death and gliosis (Figure 4); P75, pre-symptomatic in the early stages of gliosis (Figure 4), but before significant motor neuron loss; symptomatic and at onset of hindlimb paralysis when both gliosis and motor neuron loss are extensive (mean P127).

4.1.2 Ubiquitin-Proteasome Dysfunction Leads to Increased Expression of Ubiquitinated Proteins and Altered Solubility of Mutant SOD1

In order to obtain evidence of malfunction of the ubiquitin-proteasome pathway, levels and localization of mutant SOD1 (Figure 5A) and poly-ubiquitinated proteins (Figure 5B), proteasomal substrates were detected in spinal cord sections of $SOD1^{G93A}$ and $SOD1^{WT}$ transgenic mice through the course of the disease pathogenesis. Early markers of mutant SOD1 toxicity that indicate inability to clear misfolded mutant SOD1 and other proteins include detection of insoluble $SOD1^{G93A}$, ubiquitinated proteins, SDS-resistant high molecular weight complexes containing SOD1. Expression of SOD1 (Fig. 5A) and ubiquitinated proteins (Fig. 5B) was determined in mice raised in our Institution by immunohistochemical analysis of the lumbar region using specific monoclonal antibodies against SOD1 (clone SD-G6 Sigma-Aldrich) and mono and poly-ubiquitinated proteins (clone FK2, Biomol) (refer to Section 3.9 for dilutions). Expression of SOD1 (Figure 5A) and ubiquitinated proteins (Figure 5B) remained similar in motor neurons of $SOD1^{G93A}$ and $SOD1^{WT}$ transgenic mice through the course of the disease.

Whereas $SOD1^{WT}$ is for the most part soluble in non-ionic detergent, $SOD1^{G93A}$ can be isolated from spinal cord tissue in three different forms: Triton-soluble, Triton-insoluble/SDS-soluble, and SDS-insoluble, high molecular weight complexes. These chemical species were quantified in lumbar and thoracic spinal cord. The relative amounts of these forms in spinal cord segments of $SOD1^{G93A}$ transgenic mice and $SOD1^{WT}$ transgenic mice was assessed by SDS-PAGE/Western blotting of Triton-soluble tissue extracts and Triton-insoluble pellets solubilized by SDS/mercaptoethanol containing sample buffer (refer to Section 3.4). Thirty-fold more Triton-insoluble protein was loaded onto gels relative to Triton-soluble extract. An average of 11% of SOD1 in the lumbar spinal cords of three symptomatic $SOD1^{G93A}$ transgenic mice was SDS-insoluble compared to approximately 1.3% in three age-matched $SOD1^{WT}$ transgenic mice, (Figure 6A), representing a 10 fold enrichment of insoluble SOD1 in lumbar spinal cords of $SOD1^{G93A}$ transgenic mice relative to $SOD1^{WT}$ mice. There was no significant difference in the amount of soluble SOD1 present extracted from these tissues (Fig. 6A). The SDS-insoluble, high molecular weight SOD1-immunoreactive bands, first reported by Kopito's group (Johnston et al., 2000), as well as Triton-insoluble/SDS-soluble (monomeric SOD1 on Western blots of Triton-insoluble extract) and ubiquitinated proteins, were enriched in lumbar spinal cord tissue from $SOD1^{G93A}$ transgenic mice, relative to thoracic spinal cord tissue (Fig. 6B). None of these ubiquitinated species were observed in insoluble tissue from to $SOD1^{WT}$ mice and non-transgenic littermates (data not shown). These results point to inadequate clearance of modified proteins, including SOD1 in lumbar spinal cord of $SOD1^{G93A}$ transgenic mice, but a lower load of abnormal mutant SOD1 substrate in thoracic cord. These results parallel the higher vulnerability and involvement of lumbar spinal cord cells to the disease process. Also, a preferential accumulation of poly-ubiquitinated proteins, substrates of the 26S proteasome in the lumbar spinal cord of $SOD1^{G93A}$ transgenic

mice suggests that the ubiquitin-proteasome pathway is overloaded and possibly impaired in the lumbar region of the spinal cord.

4.2 Proteasome Impairment in *in vivo* and *in vitro* models of ALS

Ubiquitinated inclusion bodies in astrocytes and motor neurons in animal models of fALS as well as in spinal cord sections from sporadic and fALS patients suggest that the ubiquitin-proteasome pathway might play a role in ALS pathophysiology. Levels of mutant SOD1, but not WT SOD1 are elevated upon treatment with proteasome inhibitors suggesting that mutant SOD1 is degraded by the proteasome and increased levels of mutant SOD1 might overload its activity (Hoffman et al., 1996; Johnston et al., 2000). Also motor neurons from primary spinal cord cultures were found to be preferentially vulnerable to low-level treatment with proteasome inhibitors indicating that proteasome impairment over time can lead to motor neuron degeneration (Urushitani et al., 2002). Further *in vitro* reports studying the proteasome activity in cell lines stably and transiently overexpressing mutant SOD1 have produced contradictory findings, showing total proteasomal activity to be decreased (Hyun et al., 2002b; Urushitani et al., 2002; Allen et al., 2003), increased (Casciati et al., 2002; Aquilano et al., 2003) or unchanged (Lee et al., 2001). However, these studies did not report specific activities. Differences in physiological properties of clonal cell lines, including ability to upregulate proteasomes or other protective mechanisms may have contributed to disparate measures of activity. In the experiments described in this section, the three proteolytic activities were measured in cell lines overexpressing $SOD1^{G93A}$ and $SOD1^{G41S}$ and were normalized to levels of the structural α subunits of the proteasome to derive the specific proteasome activities in these cell lines. Also to test the hypothesis that dysfunction of the proteasome contributes to ALS pathogenesis, specific proteasome activities were measured in CNS area most affected by the disease (lumbar spinal cord), in a less affected CNS region

(thoracic spinal cord) and in non-affected tissue (liver) at various stages of disease pathogenesis in a transgenic line of mice overexpressing $SOD1^{G93A}$, a well characterized model of fALS.

Experimental Approach and Results

4.2.1 Early, Focal Reduction of Proteasomal Activities in Lumbar Spinal Cord of $SOD1^{G93A}$ Transgenic Mice

Proteasomal activities were measured in lumbar spinal cord, thoracic spinal cord (Figure 7A,C,D upper and middle panels) and liver (Figure 7A,C,D lower panels) from $SOD1^{G93A}$ and $SOD1^{WT}$ transgenic mice and their respective LM in the three stages of disease pathogenesis described in Section 4.1.1: P45, prior to motor neuron death and gliosis (Figure 4); P75, in the early stages of gliosis (Figure 4), but before significant motor neuron loss; and at onset of hindlimb paralysis when both gliosis and motor neuron loss are extensive (P115-P136, mean P127 days) (Chiu et al., 1995; Hall et al., 1998; Alexianu et al., 2001). Since no ATP was added to the homogenizing buffer, the majority of proteasomal species must have been unassembled 20S complexes (see native gel results in Chapter 5).

Assays were based on hydrolysis of fluorogenic substrates specific for each of the chymotrypsin-like, caspase-like and trypsin-like activities of the proteasome. At day 45, total chymotrypsin-like activity of the proteasome, calculated as nmol/min/mg normalized to actin, was significantly impaired in the lumbar spinal cords, at 67% of LM activity (Figure 7A top panel). The lumbar region of the spinal cord contains cell bodies of motor neurons that degenerate during the course of the neurological disease in $SOD1^{G93A}$ transgenic mice (Chiu et al., 1995f). By 75 days of age, all three major activities of the proteasome were significantly reduced, with chymotrypsin- (Figure 7A top panel), caspase- (Figure 7C top panel) and trypsin-like (Figure 7D top panel) activities in $SOD1^{G93A}$ mice being 47%, 66%,

and 54%, respectively, of the activities measured in LM controls. Chymotrypsin- (Figure 7A top panel), caspase- (Figure 7C top panel), and trypsin-like (Figure 7D top panel) activities in lumbar spinal cord from symptomatic animals (median: day 126) were 60%, 67%, and 63% of LM control activities, respectively. No significant impairment in any total proteasome activity was measured in thoracic spinal cord (Figure 7A,C,D upper panel) or liver (Figure 7A,C,D lower panels) of $SOD1^{G93A}$ transgenic mice, tissues previously shown to exhibit limited pathological changes (Chiu et al., 1995). Nor were any proteasomal activities significantly decreased in any tissue from $SOD1^{WT}$ transgenic mice in comparison to respective LM controls (Figure 7A,C,D middle and lower panels). In summary, an early focal reduction of total proteasome activity was measured within the lumbar region of the spinal cord of $SOD1^{G93A}$ transgenic mice, which preceded both markers of gliosis and clinical symptoms.

4.2.2 Reduction in Proteasome Specific Activities in Lumbar Region of Spinal Cord of $SOD1^{G93A}$ Transgenic Mice

To assess whether the observed reduction in total proteasome activity in lumbar spinal cord of $SOD1^{G93A}$ mice was the result of decreased concentration of proteasomes or reduction in specific activity, the levels of the strucutural α subunits of the 20S proteasome in all samples were examined by Western blotting, using actin as a loading control (Fig. 7B). 20S α subunits were quantified as a measure of total proteasome concentration because they are non-catalytic, structural components of all proteasomes, and in contrast to β-subunits, do not change in response to interferon-γ (Baumeister et al., 1998; Rock and Goldberg, 1999; Preckel et al., 1999; Goldberg et al., 2002). Microglial activation in the spinal cord induces over-expression of interferon-γ (Hensley et al., 2003), which could lead to a replacement of 20S β-subunits by the inducible β_i immunoproteasome subunits and subsequent changes in proteasomal degradation of substrates (Chiu et al., 1995; Gaczynska et al., 1996; Rock and

Goldberg, 1999). A representative blot is shown in Figure 7B. Expression of 20S α-subunits in the lumbar spinal cord region of $SOD1^{G93A}$ or $SOD1^{WT}$ transgenic animals was similar to that in LM controls at all ages tested. Transgenic animals and LM controls for a given tissue (e.g., lumbar spinal cord, thoracic cord, or liver) were processed on the same gel for normalization.

Total proteasomal activity (nmol/min/mg shown in Fig. 7A,C,D) were normalized to the amount of 20S α-subunits quantified on Western blots (Figure 8A,C,D). Chymotrypsin-, caspase- and trypsin-like activities of $SOD1^{G93A}$ mice normalized to 20S proteasome α-subunits levels were respectively 71%, 81%, and 91% of LM controls at day 45; 39%, 56% and 45% at day 75, and 62%, 69% and 65% at the symptomatic stage (Fig. 8A,C,D upper panels). These results followed the same pattern as total proteasomal activities and the reductions were statistically significant at day 75 and day 120. These data indicated that the decrease in total proteasome activity observed in lumbar spinal cords of $SOD1^{G93A}$ mice was due to changes in the catalytic properties of the proteasome and not due to the overall proteasome levels.

4.2.3 Proteasome Activity is Reduced in Lumbar Spinal Cord of $SOD1^{G93A}$ mice in Comparison to $SOD1^{WT}$ mice

In the preceding results, data obtained using tissues from $SOD1^{G93A}$ mice and their LM were compared. To control for over-expression of human SOD1, data obtained using $SOD1^{G93A}$ mouse tissues were compared to measurements in the same tissue from $SOD1^{WT}$ mice. Similar reductions of specific activities of the proteasome in the lumbar region of spinal cord from $SOD1^{G93A}$ were observed upon comparison to data from $SOD1^{WT}$ mice (Figure 9). Within a litter, mean proteasome specific activity of lumbar spinal cords from transgenic animals was divided by the mean specific activity of LM controls. Normalizing the activities

for the two lines of transgenic animals to their littermates eliminates the effects of genetic background. At day 45, mean specific chymotrypsin-like activity was significantly reduced in lumbar spinal cord of $SOD1^{G93A}$ mice to 73% of $SOD1^{WT}$ activity (Figure 9A). At day 75 mean chymotrypsin-, caspase-, and trypsin-like activities of lumbar spinal cords of $SOD1^{G93A}$ transgenic mice, were 36%, 53%, and 38% of $SOD1^{WT}$ activity, respectively (Figure 9A,B,C). Similar significant reductions were measured at symptomatic age (Figure 9A,B,C).

In thoracic spinal cord, there were no significant differences in total proteasomal activities in either $SOD1^{G93A}$ or $SOD1^{WT}$ transgenic mice in comparison to their respective LM controls (Figure 7A,C,D top and middle panels). However, after normalization to LM controls the chymotrypsin-like activity in thoracic cord of symptomatic $SOD1^{G93A}$ was significantly lowered relative to $SOD1^{WT}$ (Fig. 9D). This could represent late developing, disease-related functional impairment or could be due to the increase in proteasome activity of $SOD1^{WT}$ relative to their LM (Fig. 7A,C,D middle panel).

4.2.4 Proteasome Inhibition upon Stable Expression of Mutant SOD1 in NIH-3T3 Cell Lines

Total and proteasome-normalized chymotrypsin-, caspase-, and trypsin-like activities of the proteasome were impaired significantly in NIH 3T3 cells stably expressing $SOD1^{G93A}$ and $SOD1^{G41S}$ in comparison to either $SOD1^{WT}$ expressing or untransfected cells (Figure 10). The caspase-like activity of the $SOD1^{WT}$ line was significantly increased to 130% of untransfected NIH 3T3 cells (Figure 10E), possibly a general effect of the overexpression of proteins (Hyun et al., 2002a). Proteasome-normalized mean chymotrypsin-, caspase-, and trypsin-like activities were reduced to 27%, 36%, and 55% of untransfected cells, in $SOD1^{G93A}$ expressing cells, and 28%, 40%, and 55% in those expressing $SOD1^{G41S}$ (Figure 10 D,E,F). Thus overexpression of different fALS associated ($SOD1^{G93A}$ and $SOD1^{G41S}$) mutant SOD1 led to impaired proteasomal activity in NIH 3T3 cell lines.

4.2.5 Decreased Expression of the Proteasome in Motor Neurons of Lumbar Spinal Cord of SOD1^{G93A} Mice

Motor neurons generally expressed high levels of 20S proteasomal α subunits compared to the surrounding neuropil (Fig. 11A) in agreement with a previously published report (Mengual et al., 1996). However, immunolabeling for α subunits of the 20S proteasome was considerably reduced in lumbar spinal motor neurons of symptomatic SOD1^{G93A} transgenic mice compared to motor neurons of LM and SOD1^{WT} transgenic mice (Fig. 11A). This was not due to a motor neuron specific and generalized reduction in protein expression because motor neurons still labeled strongly by an antibody directed against murine NF-L and the neuropil levels appeared similar in sections of P45 and symptomatic SOD1^{G93A} transgenic mice (Figure 11B). These results demonstrate a motor neuron-specific loss of 20S proteasome expression within the lumbar spinal cord of SOD1^{G93A} mice.

4.2.6. Increased Expression of Hsp70 in Lumbar Spinal Cord of SOD1^{G93A} Mice

It was previously demonstrated that expression of Hsp70, but not Hsp40 is rapidly up-regulated upon treatment with low levels of proteasome inhibitors in primary spinal cord cultures in glia, but not in motor neurons (Batulan et al., 2003). Increased mRNA and protein expression of Hsp70 has been measured in primary cortical neurons upon treatment with moderate, non-toxic levels of proteasome inhibitors as well as in neurons of the substantia nigra upon unilateral infusion of lactacystin in this CNS region (Rideout et al., 2005; Ahn and Jeon, 2006). Expression of two protein chaperones, Hsp40 and Hsp70 were determined in lumbar and thoracic tissue extracts of P75 SOD1^{G93A} mice and LM. Levels of Hsp70 were significantly increased specifically in the lumbar, but not in the thoracic region, of the spinal cord of P75 SOD1^{G93A} mice (Figure 12A). These results provide indirect evidence that the

proteasome activity is impaired specifically in the lumbar region of the spinal cord of P75 $SOD1^{G93A}$ mice, since Hsp70 up-regulation might reflect a certain level of proteasome inhibition.

4.3 Discussion

Results presented in this chapter demonstrate impaired proteasome function in cell line and transgenic mouse models of familial ALS due to mutations in the *SOD1* gene. The major findings were:

(1) In transgenic mice expressing the disease-causing mutant protein, $SOD1^{G93A}$, proteasomal activity relative to LM was reduced as early as 45 days of age, indicating that abrogation of proteasomal function is involved early in pathogenesis, not as a late consequence of disease. (2) Reduction in activity occurred in tissues most affected in the disease. At presymptomatic stages, reduction in activity was measured in lumbar spinal cord, but not in thoracic spinal cord or liver, tissues showing limited pathological changes (Chiu et al., 1995). Significant differences in proteasomal activity of thoracic spinal cord were only apparent in symptomatic mice, an observation consistent with their relative preservation. (3) Both total and specific proteasomal activities were reduced to a similar extent in lumbar spinal cord of $SOD1^{G93A}$ transgenic mice. Thus, the reduced rate of proteasomal hydrolysis was not due to reduction in the amount of proteasome in the tissue or cell extract, but to rather impaired proteasome function. (4) Compounding regional reduction in specific proteasomal activity, the amount of proteasome progressively decreased in surviving motor neurons within the lumbar spinal cord of $SOD1^{G93A}$ mice relative to the surrounding neuropil. (5) No significant changes in proteasomal activity were measured in spinal cord or liver from $SOD1^{WT}$ transgenic mice. This line of mice does not develop ALS-like clinical disease, although mild vacuolar changes

in spinal motor neurons has been noted (Dal Canto and Gurney, 1995). (6) Reductions in both total and specific proteasomal activities were measured in NIH 3T3 cell lines stably expressing $SOD1^{G93A}$ or $SOD1^{G41S}$.

Motor impairment and neuropathological changes in the B6SJL-TgN($SOD1^{G93A}$)1Gur line of transgenic mice have been extensively documented and a similar time course of phenotypic presentation was observed in the present study. Motor neuronal loss occurs between 80-90 days of age (Chiu et al., 1995) and mutant SOD1- positive inclusions were visible in sections of lumbar spinal cord by immunhistochemistry at day 112 (Watanabe et al., 2001) and were isolated from symptomatic mice using a filter-trap assay (Wang et al., 2002). Early, pre-symptomatic changes include the presence of detergent-insoluble, high molecular weight SOD1 species on spinal cord from 30-day-old mice (Johnston et al., 2000; Wang et al., 2002; Puttaparthi et al., 2003) vacuolation of mitochondria in ventral root axons at 37 days of age and motor neuronal perikarya at 45 days of age (Chiu et al., 1995), upregulation of ICAM-1 and the immunological markers, IgG and FcγRI, in ventral spinal cord of 40-day-old mice (Alexianu et al., 2001) and markers of microglia and reactive astrocytes between 75-80 days of age (Hall et al., 1998; Alexianu et al., 2001; Batulan et al., 2003) (Fig. 4).

Reduction of proteasomal activity in homogenates of lumbar spinal cord occurred with a time course similar to other early pathological markers (see above), before reported motor neuron loss, and did not reflect a change in the cell-type composition. Relative to non-transgenic LM controls, significant reduction in total chymotrypsin-like activity (Fig. 7A) occurred in lumbar spinal cord by 45 days of age, before gliosis, and peaked at 75 days (Fig. 7A,B,C), an early stage of microglial/astrocytic activation (Figure 4).

Motor neurons comprise only a small percentage of the lumbar spinal cord. Thus, approximately 50% reduction in all three proteasomal activities in homogenates of lumbar spinal cord implies a regional impairment of proteasomal function involving multiple cell

types. Dysfunction of the proteasome in the surrounding non-neuronal cells could play a significant role in the death of motor neurons, since expression of mutant SOD1 exclusively in neurons may not be sufficient to produce a phenotype in transgenic mice (Pramatarova et al., 2001; Howland et al., 2002; Lino et al., 2002; Clement et al., 2003). Although cells can survive partial inhibition of proteasomal activities, as measured using peptide substrates (Ding et al., 2003), cells with the lowest margin of proteasomal capacity relative to substrate load will be most vulnerable to additional stress. Given the high sensitivity of motor neurons to peptide proteasomal inhibitors, motor neurons are the cells most likely to succumb to levels of proteasomal inhibition sufficient to disrupt protein catabolism and homeostasis (Urushitani *et al.* 2002; Kabashi *et al.* unpublished results).

The present study has defined the role of the proteasome in ALS pathogenesis by evaluating mutant SOD1's effect on both proteasomal level and activity in an affected tissue and relating changes to the known time course of neuropathology in a transgenic mouse model. To obtain measures of specific proteasomal activity, total activities in tissue/cell culture extracts (i.e., normalized to actin) were normalized to the level of 20S α-subunits, structural components of all proteasomal complexes. Previous studies have shown that the levels of 20S α-subunits assessed by Western blotting are representative of total proteasome content, being proportional to the increase in 20S/26S proteasome activity in conditions associated with induction of proteasomes (Wing et al., 1995; Medina et al., 1995; Attaix et al., 1999; Fang et al., 2000; Hasselgren et al., 2002). In the present study, overall levels of 20S α subunits in both mouse lumbar spinal cord and NIH 3T3 cell lines expressing mutant SOD1 were comparable to LM (Fig. 7B), and the three major activities of the proteasome remained reduced after normalization to 20S α-subunits. The reductions in total and specific proteasomal activities were similar, indicating abrogation of proteasome function, rather than a decrease in the amount of proteasome.

The coordinate reduction in chymotrysin-like, trypsin-like and caspase-like activities indicates that proteasomal cleavage of substrates was being measured, not hydrolysis by other non-proteasomal enzymes in the extract. Supporting this conclusion is that similar results were obtained using either lactacystin or MG132 to define total proteasome activity. These inhibitors have different affinities for non-proteasomal enzymes: MG132 also inhibits calpains and cathepsins, whereas the more specific proteasomal inhibitor, lactacystin, also inhibits cathepsin A (Kisselev and Goldberg, 2001).

Despite consistent expression of 20S α subunits in extracts of lumbar spinal cord measured by Western blotting, immunohistochemical analysis of tissue sections revealed substantial reduction in labeling of lumbar motor neurons relative to the surrounding neuropil in symptomatic $SOD1^{G93A}$ mice. Although a recent report indicates activities of proteasomes in spinal cord are relatively low compared to other tissues (Puttaparthi *et al.* 2003), proteasome levels are normally high in motor neurons relative to the surrounding neuropil (Fig 11) (Mengual et al., 1996). The high level expression of proteasomes and the high sensitivity of motor neurons to proteasome inhibitors (Urushitani *et al.* 2002; Kabashi *et al.* unpublished results) would indicate that these cells normally have a large substrate load. As motor neuron disease develops in the $SOD1^{G93A}$ mice, not only are lumbar motor neurons located in a region with impaired proteasome function, they are affected disproportionately because they lose proteasome expression relative to surrounding cells.

High-molecular weight SOD1 complexes that are prominent in spinal cord tissues of $SOD1^{G93A}$ transgenic mice very early in disease pathogenesis demonstrate a failure of protein "quality control" systems to properly dispose of misfolded proteins and suggest a central role in ALS pathogenesis of these quality control systems. The results presented here demonstrate that these high-molecular weight SOD1 species are more prominent in the lumbar region of the spinal cord pre-symptomatically relative to the adjacent thoracic tissue (Figure 6A). Also,

several of these proteins might be proteasomal substrates since they label positively to an antibody that recognizes ubiquitinated proteins (Figure 6B). These results demonstrate in vivo that misfolded, SDS-insoluble, SOD1 species are proteasomal substrates and a failure and/or overload of the ubiquitin-proteasome pathway results in their accumulation in affected regions. These data suggest that protein misfolding and failure of protein chaperones and ubiquitin-proteasome pathway play a central role in ALS pathogenesis.

In fALS and other familial neurodegenerative diseases, the ability of cells to prevent accumulation or aggregation of mutant protein and to withstand toxicity would depend upon their ability to activate or induce protective pathways, including protein chaperones and proteolytic pathways (see Section 1.2.6). Our previous studies showed presymptomatic reduction in protein chaperoning activity specifically in lumbar spinal cord of another line of $SOD1^{G93A}$ transgenic mice (Bruening et al., 1999). In this study, proteasomal activities decreased in lumbar spinal cord of $SOD1^{G93A}$ mice. Moreover, proteasome levels decreased in lumbar motor neurons relative to surrounding cells. Motor neurons are impaired in stress-induced upregulation of heat shock proteins with chaperoning activity. This results from lack of activation of the major heat shock transcription factor, HSF1, subsequent to its binding to heat shock promoter elements (Batulan et al., 2003). Interestingly, the protein levels of Hsp70, but not Hsp40, were also selectively elevated in the lumbar region of the spinal cord pre-symptomatically in $SOD1^{G93A}$ transgenic mice. Hsp70 is not a proteasomal substrate *per se*, but several studies have demonstrated that Hsp70 mRNA and protein levels are rapidly up-regulated following low levels of proteasomal inhibition in cell lines and animal tissues (Batulan et al., 2003; Ahn and Jeon, 2006), including astrocytes but not motor neurons of primary spinal cord cultures (Batulan et al., 2003). These results suggest that increased expression of several protein chaperones as well as a specific elevation of proteasomal activity is required in the affected tissue (lumbar spinal cord) in the affected cell types (motor

neurons) in order for them to properly cope with the increased load of misfolded proteins during the course of ALS pathogenesis. Alternatively, other molecular mechanisms could lead to increases in Hsp70, such as ER stress. More research is needed to determine whether strategies to specifically target the ubiquitin-proteasome pathway and protein chaperones are beneficial in fALS animal models.

Figure 4

Astroglial and microglial markers of disease progression in ventral horn of the lumbar spinal cord from SOD1^{G93A} transgenic mice

Twenty μm frozen cross-sections were labeled with antibody to (A) GFAP or (B) MAC-1 as markers of activated (A) astrocytes and of (B) microglia, respectively. Labeling was absent or minimal at day 45, evident in the ventral horn at day 75, and prominent throughout the lumbar cord at symptomatic ages (day 110–130) in lumbar spinal cord sections of SOD1^{G93A} transgenic mice. Scale bar = 40 μm.

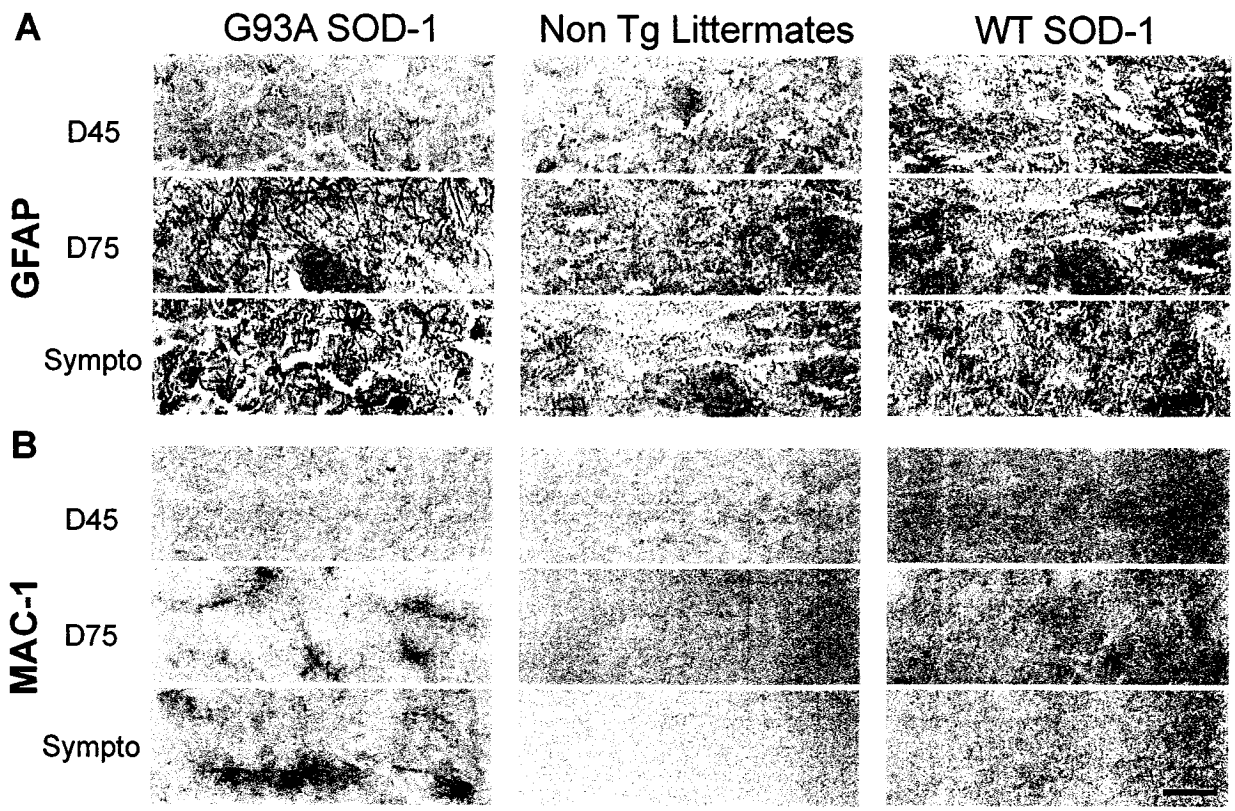

A
G93A SOD-1
Non Tg Littermates
WT SOD-1
D45
D75
Sympto
GFAP
B
D45
D75
Sympto
MAC-1

Figure 4

Figure 5

Cellular distribution of proteasomal substrates in the lumbar spinal cord of mutant SOD1 transgenic mice

Twenty μm frozen cross-sections of lumbar spinal cord from 45- and 75-day-old and symptomatic $SOD1^{G93A}$ mice, age-matched LM and $SOD1^{WT}$ transgenic mice were labeled with antibody to human SOD1 (A) and to mono-and poly-ubiquitinated proteins (B). Motor neurons were labeled strongly by both antibodies. However, no obvious changes in the localization of these proteins were observed in motor neurons throughout the course of the disease in $SOD1^{G93A}$ transgenic mice. Scale bar = 40 μm.

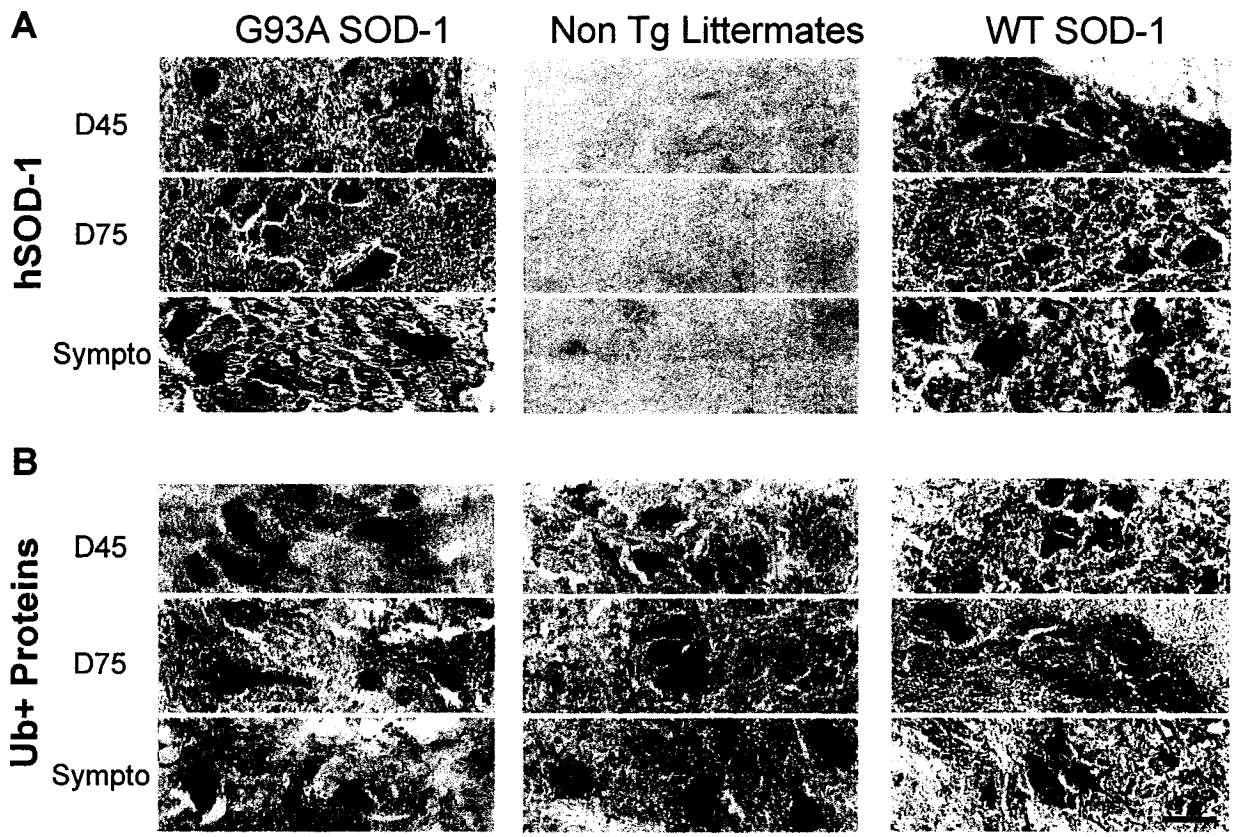

A
G93A SOD-1
Non Tg Littermates
WT SOD-1
hSOD-1
D45
D75
Sympto
B
Ub+ Proteins
D45
D75
Sympto

Figure 5

Figure 6

High-molecular weight, SDS-insoluble SOD1 species are ubiquitinated and are increased in affected tissue of SOD1^{G93A} mice

A. Even though the amount of soluble SOD1 in spinal cords of symptomatic SOD1^{G93A} transgenic mice is similar to the soluble SOD1 fraction in age-matched SOD1^{WT} transgenic mice, there is an increase of detergent-resistant SOD1 in spinal cord tissue from SOD1^{G93A} transgenic mice. Mouse tissue was homogenized in Tris/EDTA buffer as previously described, sonicated and centrifugated at 15000 g for 10 minutes. The pellets were washed three times with 0.1% TritonX in Tris/EDTA and in 0.1% SDS in Tris/EDTA and resolubilized in urea. The SDS-insoluble fraction as well as the soluble fraction were run in SDS/PAGE gels and immunoblotted using a human SOD1 specific antibody (SOD100; Stressgen). We determined a 10 fold increase of SOD1 in the insoluble fraction in the spinal cord of SOD1^{G93A} transgenic mice as compared to the SOD1^{WT} transgenic mice with no change in the soluble fraction.

B. High-molecular weight SOD1 species that are detergent-resistant are visible as early as P30 in the spinal cord of SOD1^{G93A} transgenic mice and accumulate with disease progression. Even though these species represent a very small fraction of the total insoluble SOD1, they are accumulated in the lumbar region as compared to the thoracic portion of the spinal cord as early as P75 in SOD1^{G93A} transgenic mice. We have reported a major impairment of all three activities in the lumbar region of the spinal cord early in disease pathogenesis (P75). Furthermore, the blots containing detergent-resistant proteins were re-probed with antibodies specific to ubiquitinated proteins, and a similar enrichment of the ubiquitinated species was seen selectively in the lumbar region of the spinal cord of SOD1^{G93A} transgenic mice.

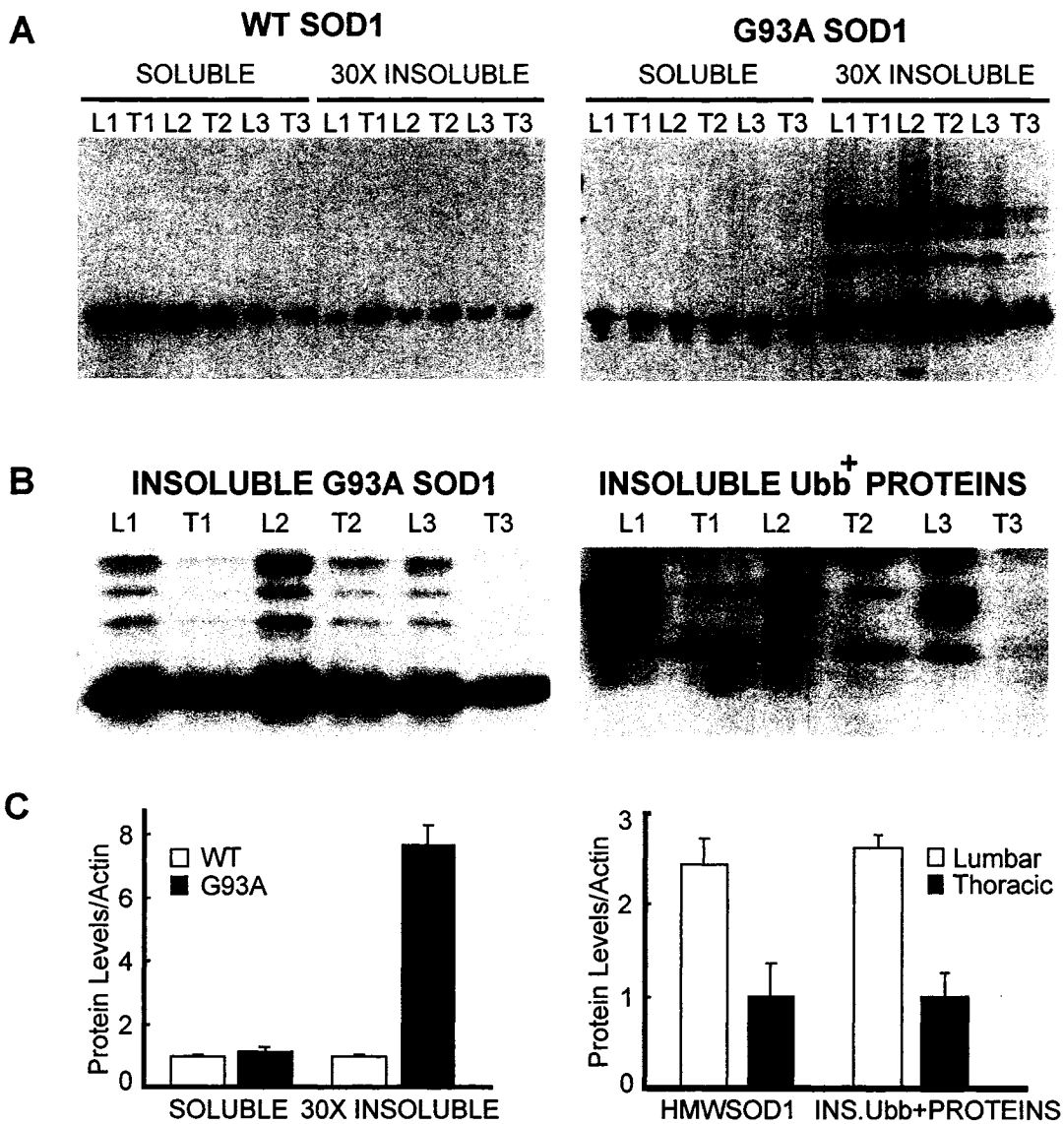

A
WT SOD1
G93A SOD1
SOLUBLE
30X INSOLUBLE
L1 T1 L2 T2 L3 T3 L1 T1 L2 T2 L3 T3
B
INSOLUBLE G93A SOD1
INSOLUBLE Ubb+ PROTEINS
L1 T1 L2 T2 L3 T3
C
Protein Levels/Actin
WT
G93A
SOLUBLE 30X INSOLUBLE
Lumbar
Thoracic
HMWSOD1 INS.Ubb+PROTEINS

Figure 6

Figure 7

Total proteasomal activities were reduced in lumbar spinal cord of $SOD1^{G93A}$ transgenic mice

Chymotrypsin-like (A), caspase-like (C) and trypsin-like (D) activities expressed as nmol/min/mg normalized to actin levels (B) were measured in homogenates of lumbar and thoracic segments of spinal cord from $SOD1^{G93A}$ (upper panels) and $SOD1^{WT}$ (middle panels) transgenic mice and compared to LM at 45 and 75 days of age and after development of symptoms (sympto; days 115-136). Lower panels show assays of liver from both lines of mice. Dark bars represent transgenic mice ($SOD1^{G93A}$ or $SOD1^{WT}$), whereas light bars represent their age-matched LM. Chymotrypsin-like activity (A) was significantly reduced at day 45 in lumbar spinal cord of $SOD1^{G93A}$ transgenics (upper panels), but not in thoracic spinal cord (upper panels) or liver (lower panels). Significant decrease in chymotrypsin- (A), caspase- (C) and trypsin-like (D) activities occurred at day 75 and at symptomatic stage, again only in lumbar spinal cord of $SOD1^{G93A}$ mice. Proteasomal activities are expressed as means ± SEM (nmol/min/mg) of four independent measurements in three to five animals per group. Significantly different from LM control: * $p<0.05$; ** $p<0.01$; *** $p<0.005$. (B) Western blots of spinal cord homogenates from $SOD1^{G93A}$ (upper blot) and $SOD1^{WT}$ (lower blot) transgenic mice and their respective LM controls labeled by antibodies against actin and 20S proteasome α-subunits.

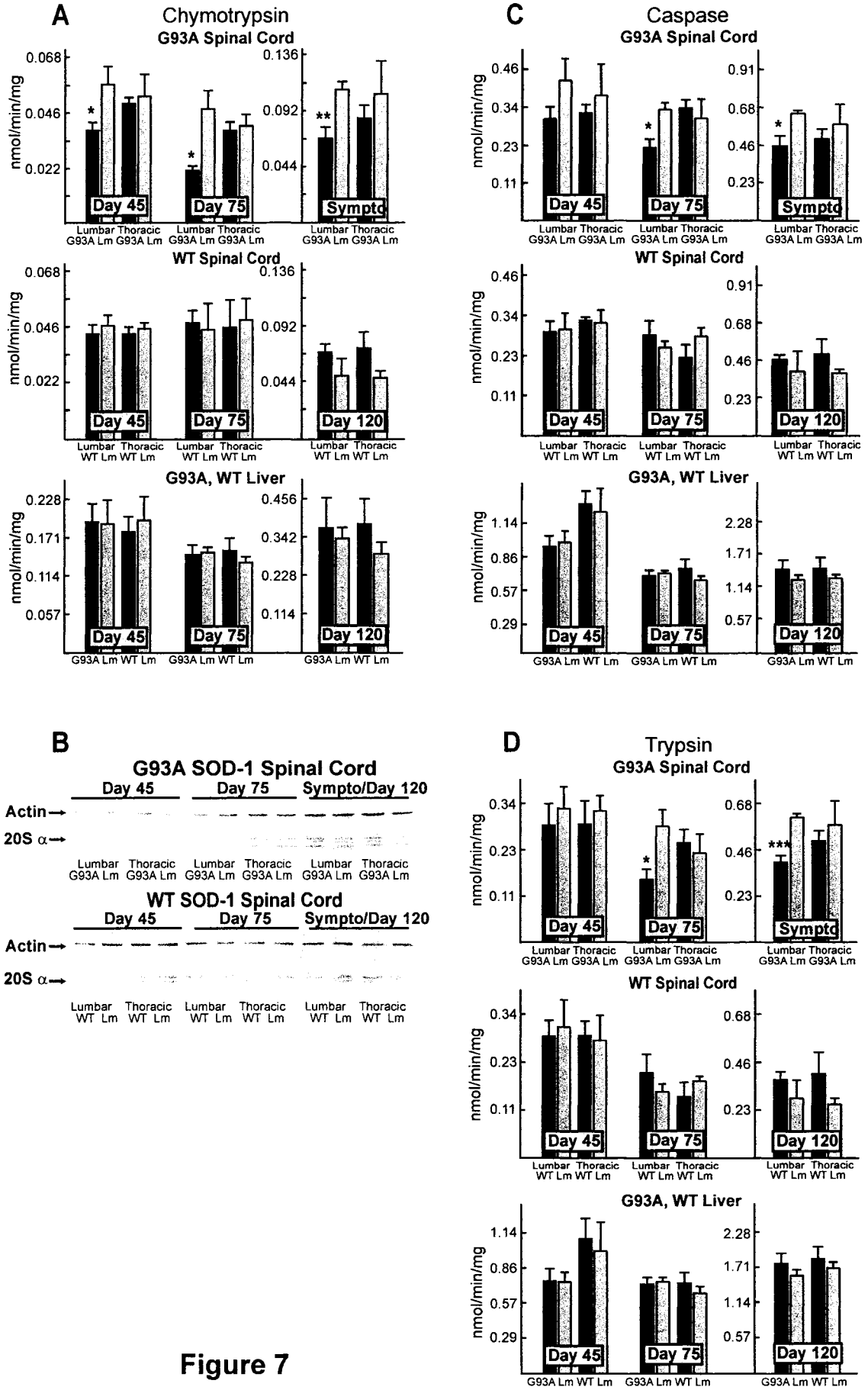

A
Chymotrypsin
G93A Spinal Cord
nmol/min/mg
0.068
0.046
0.022
0.136
0.092
0.044
Day 45
Day 75
Sympto
Lumbar Thoracic
G93A Lm G93A Lm
WT Spinal Cord
Day 120
WT Lm WT Lm
G93A, WT Liver
0.228
0.171
0.114
0.057
0.456
0.342
0.228
0.114
G93A Lm WT Lm
C
Caspase
0.46
0.34
0.23
0.11
0.91
0.68
0.46
0.23
1.14
0.86
0.57
0.29
2.28
1.71
1.14
0.57
B
G93A SOD-1 Spinal Cord
Day 45
Day 75
Sympto/Day 120
Actin
20S α
Lumbar Thoracic
G93A Lm G93A Lm
WT SOD-1 Spinal Cord
WT Lm WT Lm
D
Trypsin
0.34
0.23
0.11
0.68
0.46
0.23

Figure 7

Figure 8

Specific proteasome activities were reduced in lumbar spinal cord of $SOD1^{G93A}$ transgenic mice

Chymotrypsin-like (A), caspase-like (C) and trypsin-like (D) activities expressed as fluorescence units (F.U.) normalized to 20S α-subunit levels (B) were measured in homogenates of lumbar and thoracic segments of spinal cord from mice transgenic for $SOD1^{G93A}$ (upper panels) and $SOD1^{WT}$ (middle panels) and compared to non-transgenic littermates (LM) at 45 and 75 days of age and after development of symptoms (sympto; days 115-136). Lower panels show assays of liver from both lines of mice. Dark bars represent transgenic mice ($SOD1^{G93A}$ or $SOD1^{WT}$), whereas light bars represent their age-matched non-transgenic littermates. Significant decrease in specific chymotrypsin- (A), caspase- (C) and trypsin-like (D) activities occurred at day 75 and at symptomatic age, only in lumbar spinal cord of $SOD1^{G93A}$ transgenic mice, not in thoracic spinal cord (upper panels) or liver (lower panels). Shown are means ± SEM (nmol/min/mg) of four independent measurements in three to five animals per group. Significantly different from littermate control: * $p<0.05$; ** $p<0.01$; *** $p<0.005$. (B) Western blots of spinal cord homogenates from $SOD1^{G93A}$ (upper blot) and $SOD1^{WT}$ (lower blot) transgenic mice and their respective controls labeled by antibodies against actin and 20S proteasome α-subunits.

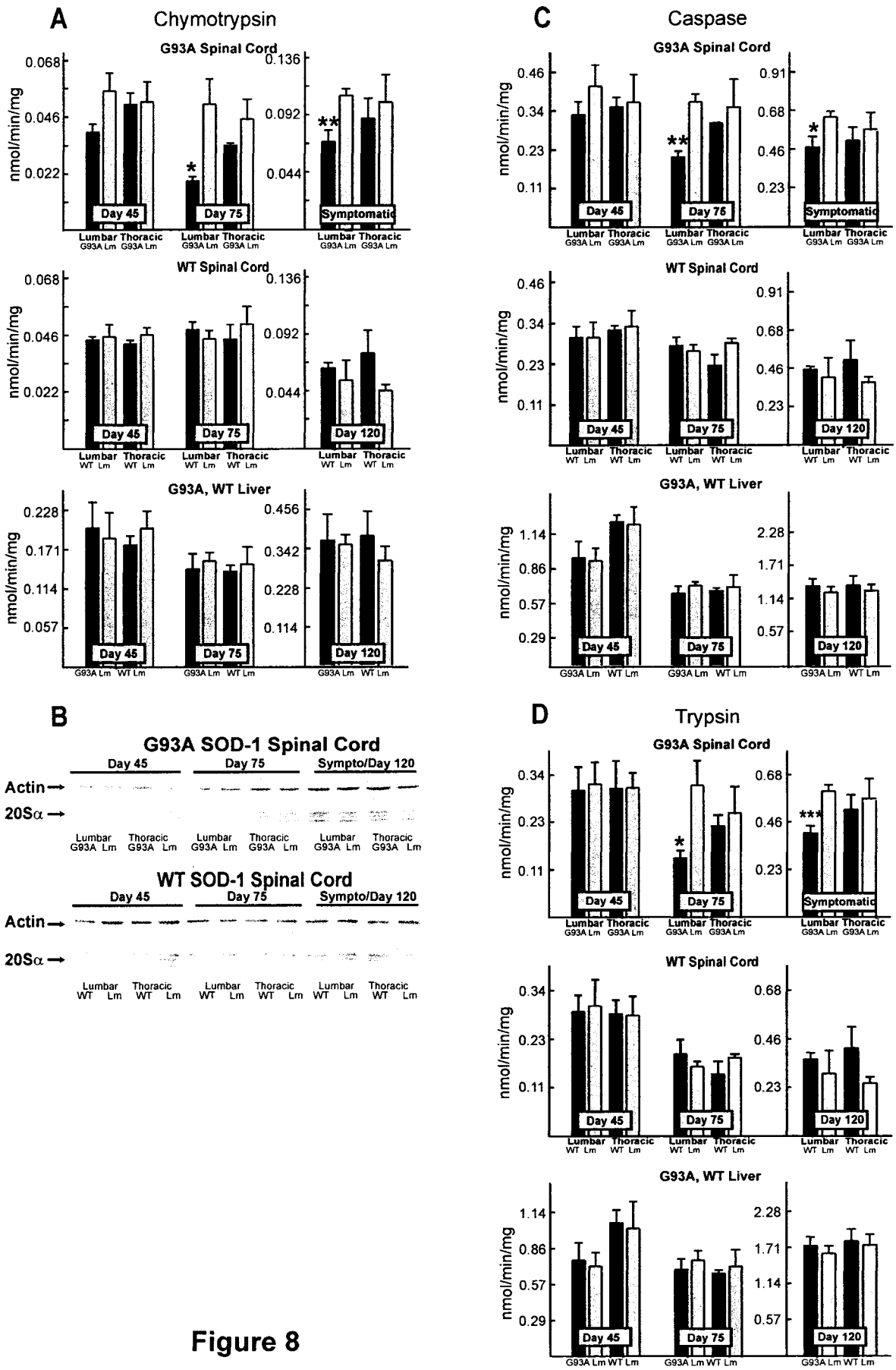

A
Chymotrypsin
G93A Spinal Cord
WT Spinal Cord
G93A, WT Liver
nmol/min/mg
Day 45
Day 75
Symptomatic
Day 120
Lumbar Thoracic
G93A Lm
WT Lm
B
G93A SOD-1 Spinal Cord
WT SOD-1 Spinal Cord
Day 45
Day 75
Sympto/Day 120
Actin
20Sα
Lumbar Thoracic
G93A Lm
WT Lm
C
Caspase
D
Trypsin

Figure 8

Figure 9

Decrease in specific activities of the proteasome in lumbar spinal cord of $SOD1^{G93A}$ relative to $SOD1^{WT}$ transgenic mice

To compare specific proteasomal activities in tissue homogenates from the two lines of mice, specific activities for tissue from each mouse were calculated by normalizing total activity in nmol/min/mg first to actin then to the levels of 20S proteasome α-subunits (see Figure 8). Then specific activities from transgene-expressing mice were expressed as percent of specific activity in LM. Shown are specific chymotrypsin- (A,D), caspase- (B,E) and trypsin-like (C,F) activities of lumbar (A,B,C) and thoracic (D,E,F) spinal cord homogenates. Shown are means ± SEM. Significant difference between $SOD1^{G93A}$ and $SOD1^{WT}$: * $p<0.05$; ** $p<0.01$; *** $p<0.005$.

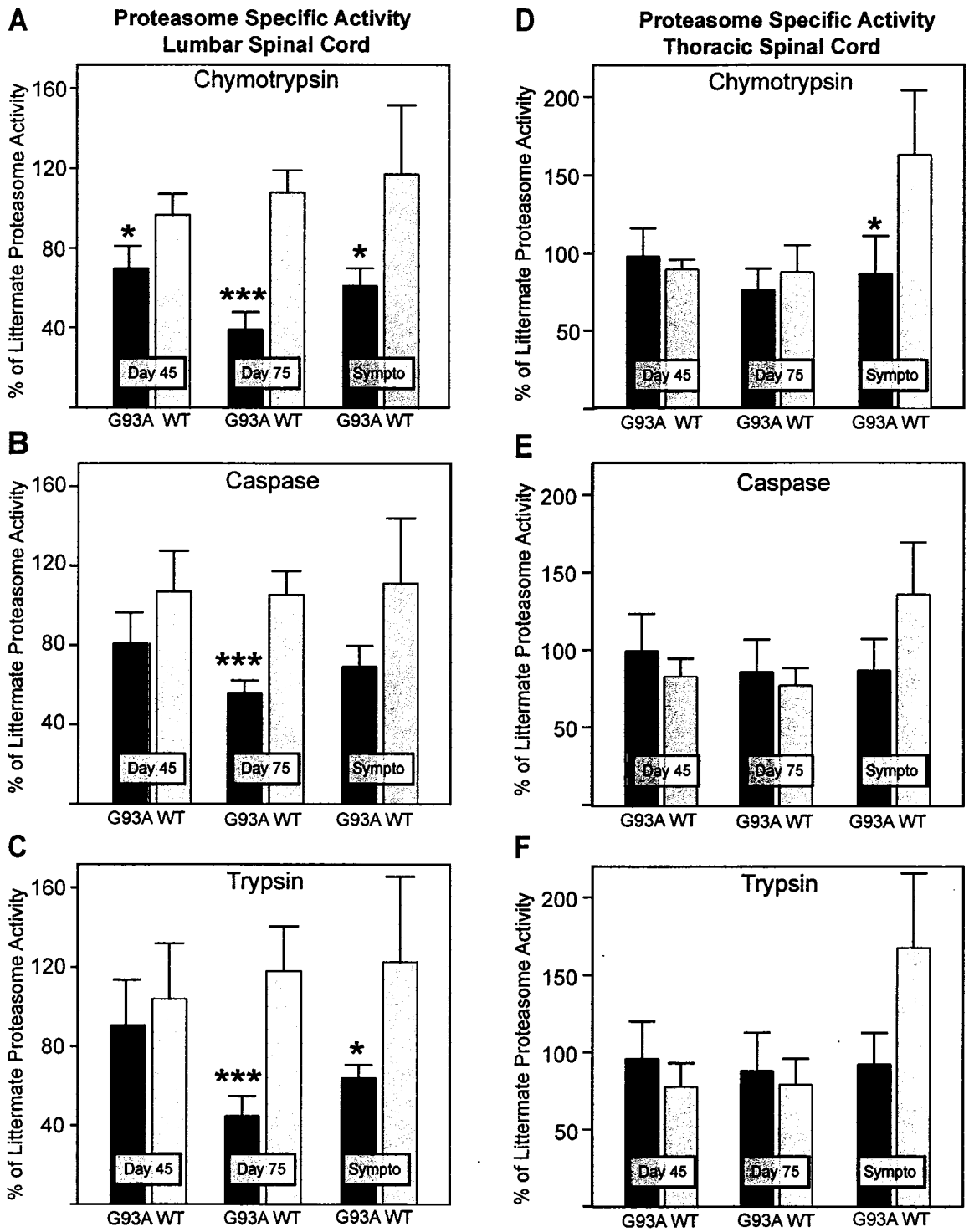

A
Proteasome Specific Activity
Lumbar Spinal Cord
Chymotrypsin
% of Littermate Proteasome Activity
160
120
80
40
*
***
*
Day 45
Day 75
Sympto
G93A WT
G93A WT
G93A WT
D
Proteasome Specific Activity
Thoracic Spinal Cord
Chymotrypsin
% of Littermate Proteasome Activity
200
150
100
50
*
Day 45
Day 75
Sympto
G93A WT
G93A WT
G93A WT
B
Caspase
% of Littermate Proteasome Activity
160
120
80
40
***
Day 45
Day 75
Sympto
G93A WT
G93A WT
G93A WT
E
Caspase
% of Littermate Proteasome Activity
200
150
100
50
Day 45
Day 75
Sympto
G93A WT
G93A WT
G93A WT
C
Trypsin
% of Littermate Proteasome Activity
160
120
80
40
***
*
Day 45
Day 75
Sympto
G93A WT
G93A WT
G93A WT
F
Trypsin
% of Littermate Proteasome Activity
200
150
100
50
Day 45
Day 75
Sympto
G93A WT
G93A WT
G93A WT

Figure 9

Figure 10

Total proteasome activities and activities normalized to proteasome levels are reduced in NIH 3T3 cell lines stably expressing mutant SOD1

Total (A,B,C: normalized to actin) and normalized to 20S α-subunits (D,E,F) chymotrypsin- (A,D), caspase- (B,E) and trypsin-like (C,F) activities of the proteasome were significantly reduced in homogenates of NIH 3T3 cells stably expressing human mutant $SOD1^{G93A}$ or $SOD1^{G41S}$ compared to untransfected NIH 3T3. Shown are means ± SEM (nmol/min/mg) of three independent cultures for each cell line with each sample analyzed in quadruplicate. Significantly different from untransfected NIH 3T3: * $p<0.05$; ** $p<0.01$; *** $p<0.005$.

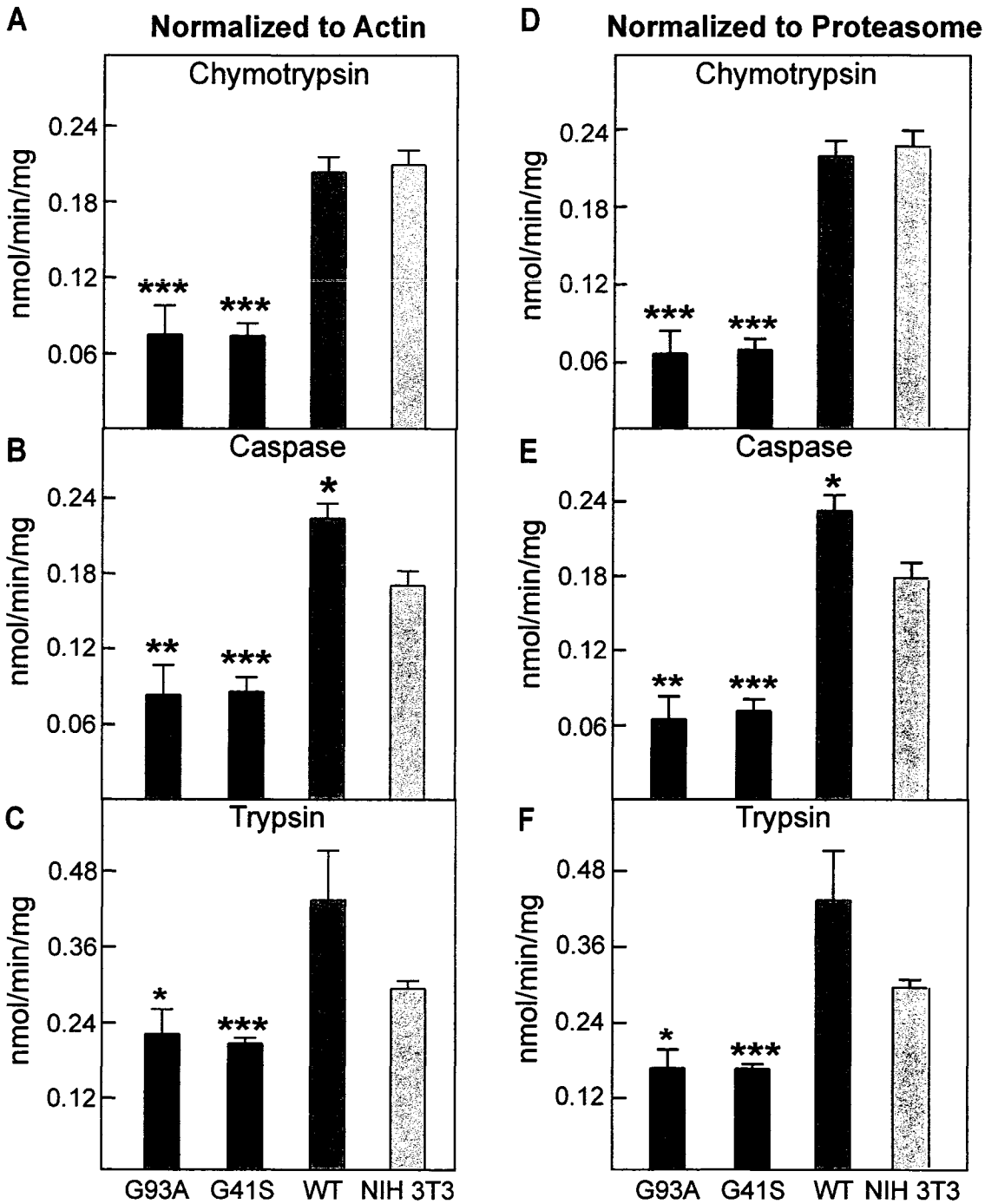

A
Normalized to Actin
Chymotrypsin
nmol/min/mg
0.24
0.18
0.12
0.06
***
***
B
Caspase
0.24
0.18
0.12
0.06
**
***
*
C
Trypsin
0.48
0.36
0.24
0.12
*
***
G93A
G41S
WT
NIH 3T3
D
Normalized to Proteasome
Chymotrypsin
E
Caspase
F
Trypsin

Figure 10

Figure 11

Levels of α subunits of the 20S proteasome in the lumbar motor neurons of mutant SOD1 transgenic mice

Lumbar spinal cord was obtained from 45- and 75-day-old and symptomatic $SOD1^{G93A}$ mice, and from age-matched littermates and $SOD1^{WT}$ transgenic mice. Twenty μm frozen cross-sections were labeled with antibody to α-subunits of the 20S proteasome core (A). Labeling of motor neurons in cord from 75-day-old and symptomatic $SOD1^{G93A}$ transgenics (arrows) appeared markedly reduced relative to the surrounding neuropil and to motor neurons of non-transgenic littermates and $SOD1^{WT}$ transgenics, despite the labeling for NF-L (B), mono- and poly-ubiquitinated proteins and human SOD1 (see Figure 4) did not change in motor neurons. Scale bar = 40 μm.

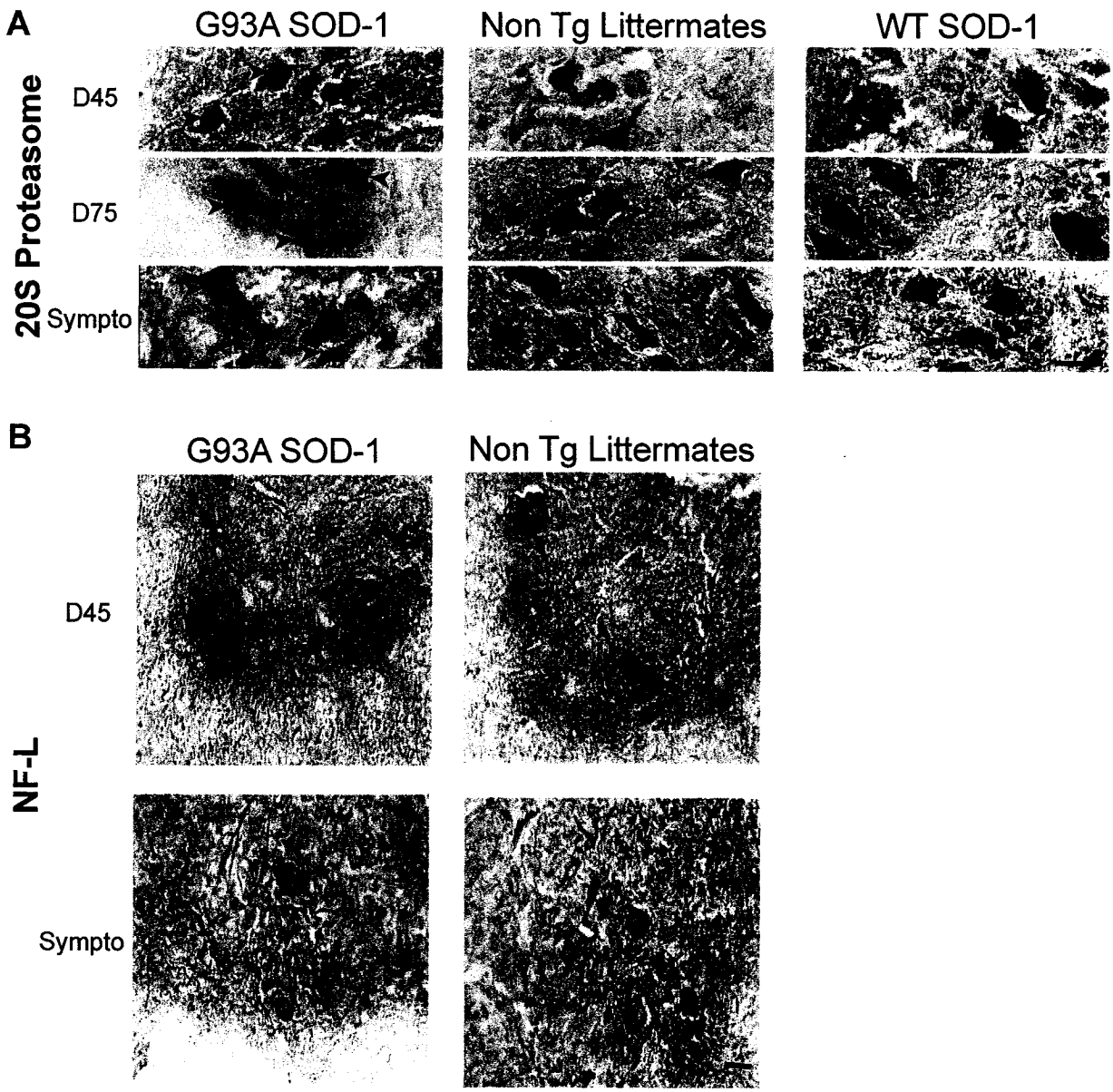

A
G93A SOD-1
Non Tg Littermates
WT SOD-1
20S Proteasome
D45
D75
Sympto
B
G93A SOD-1
Non Tg Littermates
NF-L
D45
Sympto

Figure 11

Figure 12

Elevation of Hsp70 early in pathogenesis of fALS suggests dysfunction of the UPS

A. That levels of Hsp70 are increased in lumbar, but not in thoracic, spinal cord of P75 $SOD1^{G93A}$ transgenic mice provides *in vivo* evidence of an impairment of the proteasome pathway, since levels of this protein chaperone have been found to be increased upon low levels of proteasome inhibition in primary spinal cord cultures. Levels of another protein chaperone, Hsp40, which is not up-regulated upon moderate inhibition of the proteasome using peptide inhibitors of the 20S proteasome did not change in the lumbar region of the spinal cord of P75 $SOD1^{G93A}$ transgenic mice. The levels of 20S α subunits and actin (loading control) were not changed (* $p<0.05$).

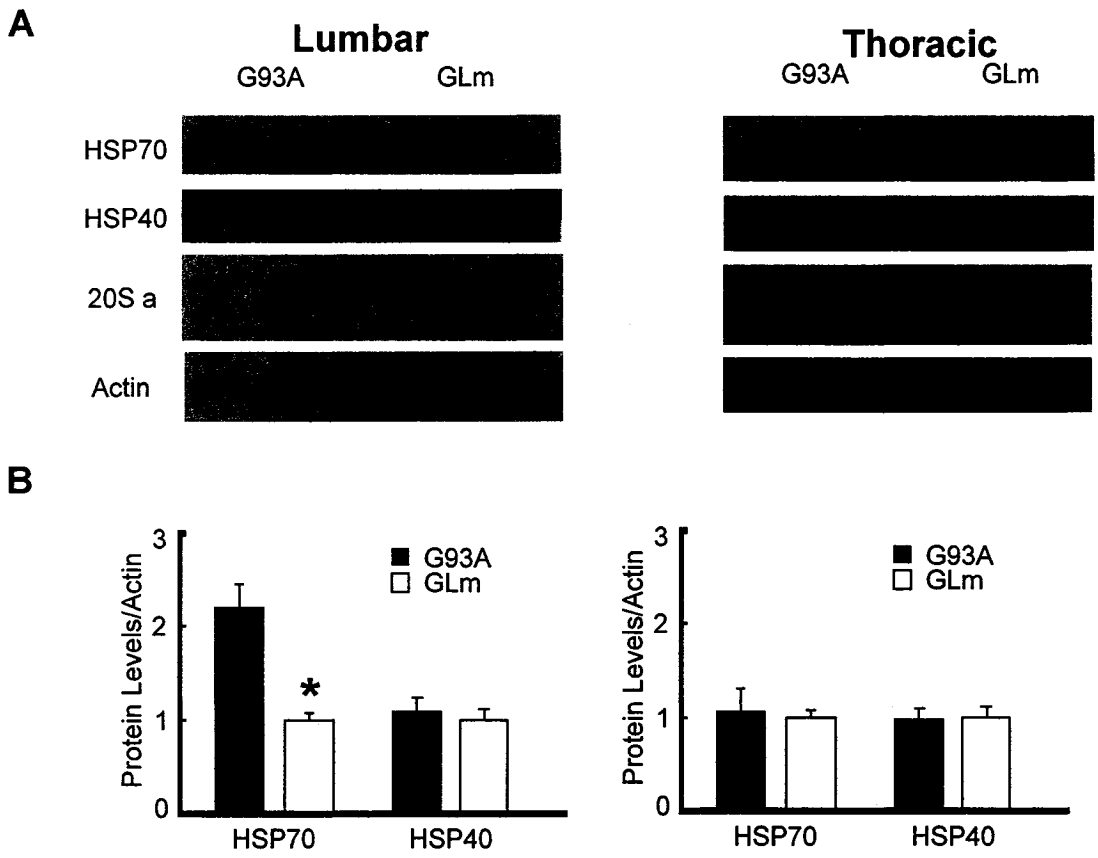

A
Lumbar
G93A
GLm
Thoracic
G93A
GLm
HSP70
HSP40
20S a
Actin
B
Protein Levels/Actin
3
2
1
0
G93A
GLm
*
HSP70
HSP40
Protein Levels/Actin
3
2
1
0
G93A
GLm
HSP70
HSP40

Figure 12

Chapter 5 Understanding the Mechanisms of Proteasome Impairment in ALS Pathogenesis

The assays of enzyme activity described in Chapter 4 measure the ability of small exogenous peptide substrates to compete with endogenous substrates in tissue homeogenates for cleavage by proteolytic acitivities of the proteasome. Thus, gradual reduction in specific chymotrypsin-, trypsin- and caspase-like proteasomal activities could reflect either changes in composition, assembly or post-translational modification of proteasome complexes or inhibition of the proteasome by a specific protein including, but not limited to, mutant SOD1. In this chapter, as stated in Specific Aim 2, the mechanisms of proteasome impairment were studied in affected tissue of the murine model of fALS, $SOD1^{G93A}$ transgenic mouse. These results have been submitted for publication to the Journal of Biological Chemistry.

5.1 Changes in Composition of Proteasome Complexes Correspond to Impairment of Proteasome Activity

Focal dysfunction of proteasome activity early in development of disease in the fALS mouse cannot be explained fully by reduction in amount of structural α subunits of the 20S proteasome. As discussed in the previous chapter, except for the late but dramatic reduction in lumbar motor neurons, levels of 20S α subunits remained constant in the examined tissues from $SOD1^{G93A}$ transgenic mice (Figure 7B). Other possibilities for proteasomal impairment would include: reduced levels of functional β subunits of the 20S core; isoform switching of catalytically active 20S β subunits (Allen et al., 2003), altered 20S:26S ratio, association of 20S with 19S or 11S regulatory subunits, or a combination (Keller et al., 2000b); impaired assembly of proteasomal subunits into active complexes, and modification (e.g., oxidation, hyperphosphorylation) of proteasomal subunits that impair enzyme activity, as documented in

ischemia-reperfusion injury (Keller et al., 2000b) and ethanol treatment (Bardag-Gorce et al., 2004).

The composition of the 20S/26S/immunoproteasome was assessed further by analyzing expression of two β subunits of the 20S core particle at the protein and mRNA levels and determining if these subunits were assembled into 20 or 26S proteasomes: 1) the catalytic subunit (β5) with predominantly chymotrypsin-like activity; thought to be the major proteolytic activity of the proteasome and the earliest reduced activity in the lumbar spinal cord of $SOD1^{G93A}$ mice; and 2) a structural β subunit (β3). Also the levels of the 19S and 11S regulatory particles of the proteasome were determined in affected and less affected CNS tissue of $SOD1^{G93A}$ transgenic mice early in disease pathogenesis as well as at symptomatic stages.

Experimental Approach and Results

5.1.1 Early Reduction in β Subunits of the 20S Proteasome in Lumbar Spinal Cord of $SOD1^{G93A}$ Transgenic Mice

In order to analyze the expression of various subunits that compose the proteasome complexes, the expression of the functional subunit harbouring chymotrypsin-like activity of the proteasome, β5 and its inducible counterpart, β5i the expression of β3, a structural subunit of the 20S core as well as subunits of the 19S and 11S regulatory particles of the proteasome were determined.by Western blot analysis in lumbar and thoracic spinal cord extracts from $SOD1^{G93A}$ transgenic mice, $SOD1^{WT}$ transgenic mice and their non-transgenic littermates at three stages of disease pathogenesis described in Chapter 4 using specific antibodies (see Section 3.9). Subunits β5 and β3 of the 20S core were substantially reduced in lumbar spinal cord of 45 day-old (Figure 13) and 75 day-old (Figure 14) $SOD1^{G93A}$ transgenic mice compared to their non-transgenic littermates (LM) and to mice transgenic for $SOD1^{WT}$. No

significant changes in these subunits were detected on Western blots of extracts of thoracic spinal cord from $SOD1^{G93A}$ or $SOD1^{WT}$ transgenic mice relative to their nontransgenic littermates (Figure 15A). Also, no changes of 19S6b subunit of the 19S regulatory particle as well as the 11S regulatory particle of the 20S core were determined in lumbar spinal cord of 45 day-old and 75 day-old $SOD1^{G93A}$ transgenic mice (Figure 15B).

The decrease in β5 immunoreactivity correlated with our previous experiments showing a decrease of specific chymotrypsin-like activity in lumbar spinal cord of $SOD1^{G93A}$ transgenic mice, but not the adjacent thoracic/cervical region at this age, and subsequent decrease in caspase/PGPH-like and trypsin-like activities by P75. Unfortunately, the commercially available antibodies to the β1 and β2 subunits that harbour these activities were not sufficiently specific to confidently assess their levels by Western blotting. An antibody commercially available from Calbiochem which recognizes several 20S β subunits was tested in spinal cord extracts and did not label any 20S proteasomal subunits at the right molecular weight (20-30 kDa).

5.1.2 Reduction in 20S β3 and β5 is not due to Substitution of Immunoproteasome Subunits

Immune reactions can be accompanied by substitution of immunoproteasomes for constitutive proteasomes. However, at presymptomatic stages of disease, the reductions in β5 were not accompanied by significant increase in amount of β5i subunit (Figures 13 and 14), nor were there significant alterations in markers of either the constitutive 19S regulatory subunit (19S6b), or of 11S (11Sα), which is substituted in immunoproteasomes (Figure 15). According to current understanding of proteasome composition, such drastic reduction in labelling of the proteolytically inactive β3 subunit would not be expected simply from a switch to immunoproteasomes. Also, P45 is prior to the reactive gliosis that characterizes latter stages of disease, although there may subtle changes in immune markers. P75 is prior to

significant motor neuron loss, but some activated microglia and astrocytes may be present. However, as expected from previous studies, lumbar spinal cord from symptomatic mice (approximately 120 days of age) showed significant upregulation of β5i and 11S, as well as decrease in β5 (Figure 16A), consistent with the prominent microglial activation at this stage.

Collectively, these data argue against the reduction in β3 and β5 being altered in presymptomatic mice because of substitution of immunoproteasome subunits.

5.1.3 Motor Neurons in the Spinal Cord Have a Progressive Decrease in Constitutive 20S β Subunits

Immunohistochemical analysis of the lumbar region of the spinal cord using specific antibodies to β3 (Figure 17A) and β5 (Figure 17B) at P45 and at P75 revealed that motor neurons express high levels of constitutive β subunits. Similarly, motor neurons label robustly using an antibody to the 20S core and antibodies directed against 20S α subunits. However, there is a progressive depletion of constitutive β subunits expression as the ALS-like disease progresses in lumbar motor neurons of $SOD1^{G93A}$ transgenic mice (Fig. 17A and B lower panels). The depletion of constitutive β subunits in the lumbar region observed by Western blotting is not observed in the immunohistochemical analysis due to certain cross-reactive higher molecular weight (60+ kDa) bands (data not shown). We and others found a similar decrease in the expression of α subunits of the 20S core in lumbar motor neurons only in the late stages of the disease (Cheroni et al., 2005). Also, no cell-specific changes in expression of 19S and 11S as well as 20S β5i at any stage of the disease in $SOD1^{G93A}$ transgenic mice as compared to their non-transgenic LM were observed (data not shown).

5.2 No Translational Dysfunction of 20S Proteasome Subunits in Lumbar Spinal Cord and Motor Neurons of $SOD1^{G93A}$ transgenic mice

As reviewed in Section 1.4.4, little is known about molecular mechanisms regulating constitutive and stress-induced expression of 20S proteasome subunits in mammalian cells. Treatment of cell lines with proteasome inhibitors as well as gene silencing of 26S subunits lead to increases in gene transcripts encoding certain proteasomal subunits (Meiners et al., 2003). However, other mechanisms appear to be involved in affected tissues of the fALS mouse, since impairment of proteasome activity led to decreased protein expression of 20S β subunits (refer to Section 5.1.1).

Experiments were conducted to determine whether the decrease in levels of 20S β subunits in lumbar spinal cord of $SOD1^{G93A}$ transgenic mice *(Section 4.1)*, or α (Figure 11) and constitutive β subunits (Figure 17) lowered specifically in motor neurons at the later stages of disease *(Section 5.2.1),* occurred due to reduced transcription of 20S proteasome genes.

20S proteasome mRNA transcripts were analyzed in extracts of lumbar, thoracic and cervical spinal cord of $SOD1^{G93A}$ transgenic mice and age-matched non-transgenic littermates at P55, an intermediate time point between P45 and P75. Following reverse-transcription (rt) amplification of mRNA, transcripts for α7, β3 and β5 were quantitated by rt-PCR using primers listed in Table 4, as described in section 3.8. To quantitate the mRNA levels of proteasome subunits specifically in lumbar motor neurons, motor neurons were collected by laser capture at P100. These experiments were conducted in collaboration with the laboratory of Dr. Denise Figlewicz, University of Michigan Ann Arbor.

Experimental Approach and Results

5.2.1 Reduction in 20S β3 and β5 Subunits in Lumbar Spinal Cord of $SOD1^{G93A}$ Transgenic Mice does not Result from Decreased mRNA Levels

To determine if the decrease in levels of constitutive β subunits of the proteasome at P45 and P75 was due to a transcriptional defect, mRNA levels were quantified by rt-PCR in extracts of lumbar, thoracic and cervical (Figure 18) spinal cord from P55 $SOD1^{G93A}$ transgenic mice and nontransgenic littermates. Primers used to amplify mRNA for 20S core subunits β3, β5, β5i and α7 and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) as control (refer to Table 4), as well as assays to determine specificity of primers in murine tissues were performed. Results normalized to GAPDH are presented in Figure 18. There was no significant decrease in mRNA encoding any of the 20S core subunits in the spinal cord of $SOD1^{G93A}$ transgenic mice compared to their nontransgenic littermates. In contrast, β5 subunit mRNA was significantly increased in the lumbar and cervical region of the spinal cord (Figure 18), β5i mRNA was increased in thoracic and cervical regions (Figure 18), and α7 mRNA was increased in the thoracic region of spinal cord from P55 $SOD1^{G93A}$ transgenic mice (Figure 18).

These results provided no evidence of defects in transcription of proteasomal genes in this early phase of pathogenesis, pointing to abnormalities of proteasome assembly and/or post-translational modification of subunits.

5.2.2 The Late Decrease in 20S Core Protein Levels in Lumbar Motor Neurons of $SOD1^{G93A}$ Transgenic Mice is not due to a Reduction in mRNA Levels

We and others have reported decreased levels of structural α subunits of the 20S proteasome in lumbar motor neurons from symptomatic $SOD1^{G93A}$ transgenic mice (Kabashi et al., 2004; Cheroni et al., 2005). To investigate if the decrease in 20S proteasome subunits that occurred with disease progression occurred at the mRNA level, 20Sα7, β3, β5 and β5i transcripts were quantified by rt-PCR in lumbar motor neurons captured by laser microdissection from lumbar spinal cord of 100 day old $SOD1^{G93A}$ transgenic mice and

nontransgenic littermates (Figure 19A) At this stage of the disease, there is documented motor neuron loss in the lumbar spinal cord of $SOD1^{G93A}$ transgenic mice (Chiu et al., 1995i), but morphologically healthy motor neurons still remain (Figure 19A,B). Immunological analysis confirmed decreased levels of 20S α subunits in lumbar motor neurons in the same tissue block as used for rt-PCR experiments, whereas levels of GAPDH (used as a control in rt-PCR experiments) appeared similar in sections from $SOD1^{G93A}$ transgenic mice and their littermates (Figure 19B).

No significant difference was found in mRNAs encoding constitutive β and α7 subunits in lumbar, cervical or thoracic motor neurons of $SOD1^{G93A}$ transgenic mice compared to their littermates. However, an increase of β5i mRNA levels was measured specifically in lumbar motor neurons, consistent with the increased protein levels detected by Western blotting (Fig. 19C). However, the data indicate that the decrease in protein levels of the constitutive β subunit and 20Sα proteins in lumbar motor neurons from symptomatic $SOD1^{G93A}$ transgenic mice did not result from decreased gene transcription or RNA stability.

5.3 Proteasome Assembly and Complex Formation in Lumbar Spinal Cord of $SOD1^{G93A}$ Transgenic Mice

As described in Section 1.4.3, the assembly of the mammalian proteasome is not well understood. Some of the β subunits need to be cleaved to proforms prior to being assembled as heptameric rings in the proteasome complex. Several steps are required for 28 monomeric α and β subunits to form the 20S core of the proteasome which assembly in mammalian cells is mediated by POMP and Hsc73 chaperone (Kruger et al., 2001; Heink et al., 2005). Also, another chaperone, Hsp90 plays an important role in assembly of mammalian 20S and 19S complexes and maintenance of the resulting 26S proteasome (Imai et al., 2003). A better

understanding of the important steps involved in the formation of the proteasome complex is required in order to better understand its role in neurodegenerative disorders and aging.

In *Drosophila* brain, decreases in proteasome activity associated with aging corresponded to decreased immunoreactivity of β5 subunit in the 26S proteasome complex (Wang et al., 2006b). However, the role that assembly and maintenance of 20S, immunoproteasome and 26S proteasome complexes play in ALS or other neurodegenerative disorders pathogenesis that are associated with dysfunctional ubiquitin-proteasome pathway has not been characterized. By the experiments described in this section, assembly and maintenance of 20S and immunoproteasome complexes were studied in the lumbar region of $SOD1^{G93A}$ transgenic mice compared to littermates.using two-dimensional Coomassie Blue native/SDS-PAGE analysis as described below. Also, formation of the 26S complexes was determined using a one-dimensional native gel approach enriched in ATP and glycerol to maintain the integrity of 26S proteasome complexes, as developed by Finley and colleagues (Elsasser et al., 2005) to study formation of proteasome complexes in yeast.

Experimental Approach and Results

5.3.1 20S and 26S Proteasome Complexes are Assembled, but Have Reduced Immunoreactivity to β subunits in the Lumbar Spinal Cord of $SOD1^{G93A}$ Transgenic Mice

To investigate if a decrease in proteasome activity reflected dysfunctional formation of the 20S/26S proteasome complexes, Blue native-SDS/PAGE was performed (Figure 20A). In the first dimension (Coomassie Blue native gel electrophoresis), protein complexes are separated according to their size, then subsequently broken into subunits in the second denaturing dimension (SDS/PAGE). By Western analysis using antibodies to specific proteasomal subunits (20Sα, β3 and β5) showed that most of these proteasomal proteins, from extract of lumbar spinal cord from either $SOD1^{G93A}$ transgenic mice or nontransgenic

littermates, ran assembled in 20S proteasome complexes, with a small amount of 20Sα migrating as monomer (Figure 20A). Although there was no significant difference in levels of assembled or nonassembled 20S α subunits in lumbar spinal cord of P75 $SOD1^{G93A}$ transgenic mice compared to littermates, levels of β5 and β3 subunits in 20S proteasome were substantially decreased in lumbar spinal cord of P75 $SOD1^{G93A}$ transgenic mice. Co-labelling of these complexes with 19S antibodies revealed that most of the proteasomal complexes were arranged as 20S core with minimal assembled 26S complexes (Figure 20A).

To examine the composition of intact 26S complexes, native gel electrophoresis was carried out in the presence of ATP and glycerol, which maintains the association of 19S regulatory particles and 20S proteasome core (Elsasser et al., 2005). A complex approximately 2.5 MDa in size was observed on Western blots of these gels probed with antibodies to α, β subunits as well as the S5a subunit of the 19S regulatory particle (Figure 20B). Co-migration of 19S5a and 20Sα confirmed 26S as the major species of proteasome present. These results were similar to those obtained with Blue native/SDS-PAGE that poorly preserves the association 20S proteasome core with regulatory particles; i.e., decrease of β5 and β3 in lumbar spinal cord tissue from $SOD1^{G93A}$ transgenic mice, but no changes in α subunits in assembled proteasomes.

5.4 No Direct Inhibition of the Proteasome by Mutant SOD1 in Lumbar Spinal Cord of $SOD1^{G93A}$ Transgenic Mice

That many tissues express high levels of transgenic $SOD1^{G93A}$, yet reduction in specific proteasome activities was predominant in lumbar spinal cord, argues against direct inhibition of the proteasome by mutant SOD1. However, certain post-translationally modified forms or small oligomers could be inhibitory substrates. The biophysical basis of altered detergent-solubility and polymerization of mutant SOD1 proteins *in vivo* remains to be identified, but is under investigation. Studies of recombinant proteins *in vitro* or transfected

cultured cells subjected to oxidizing conditions show that dimer destabilization and polymerization of monomers are promoted by disease-causing mutations, demetallation, disulphide reduction, and oxidative modifications (Urushitani et al., 2002; Rodriguez et al., 2002; Rakhit et al., 2002; Tiwari and Hayward, 2002; Cardoso et al., 2002; Strange et al., 2003; DiDonato et al., 2003; Chung et al., 2003; Elam et al., 2003; Hough et al., 2004; Rakhit et al., 2004; Zhang et al., 2004; Furukawa et al., 2004). Full biophysical characterization of mutant and WT SOD1 proteins from tissues will be required to resolve which states occur under physiological conditions and correlate with disease markers. In this section, *in vivo* association of mutant SOD1 with proteasome complexes in tissues from $SOD1^{G93A}$ transgenic mice was determined in native gels. Also, proteasome activities were determined in lumbar spinal after mutant SOD1 were immunodepleted from symptomatic $SOD1^{G93A}$ transgenic mice.

Experimental Approach and Results

5.4.1 Mutant SOD1 was not Found Associated with 20S Proteasomes in Lumbar Spinal Cord

Western blots of Blue native-SDS/PAGE were reprobed with antibody to human SOD1 in order to assess whether mutant protein remained associated with proteasome complexes. No SOD1-immunoreactivity co-migrated with 20S proteasomes (labeled with antibody to 20Sα subunits) (Figure 21A). Monomeric and high molecular weight forms of mutant SOD1 were detected in the lower molecular weight region of the blot.

Also, immunoprecipitation of mutant SOD1 from lumbar spinal cord tissue of symptomatic $SOD1^{G93A}$ transgenic mice using an antibody that preferentially binds to human SOD1 (SOD100) led to approximately 93% depletion of human SOD1 from spinal cord tissue extract as determined by Western blotting (Figure 21B). Subsequently, the three activities of the proteasome were assayed out in lumbar tissue before and after SOD1 immunodepletion.

No changes in the chymotrypsin-, caspase-, and trypsin-like activities of proteasome were measured as presented in Figure 21B. These results suggest that mutant SOD1 does not directly inhibit the proteasome by blocking the entrance and choking the 20S core as was previously hypothesized (Bennett et al., 2005).

5.5. Discussion

Protein levels of functional β5 subunit (subunit responsible for chymotrypsin-like proteolytic degradation) and structural β3 subunit of the 20S core of the proteasome were significantly decreased specifically in the lumbar spinal cord region of P45 (Figure 13) and P75 (Figure 14) $SOD1^{G93A}$ transgenic mice. These results demonstrate that impairment of proteasome activity coincides with decreased levels of 20S β subunits (refer to Section 4.2). Levels of 20S β subunits did not change in insoluble extracts of lumbar spinal cord from P75 $SOD1^{G93A}$ transgenic mice (data not shown) demonstrating that the decrease in the 20S β subunit levels was not due to their precipitation from the soluble fraction. Proteasome impairment in lumbar spinal cord early in fALS disease pathogenesis did not induce increased expression of subunits of the 20S core as suggested by cell line studies (Meiners et al., 2003). Rather, most of the subunits of the ubiquitin-proteasome that were quantified were not changed, whereas levels of 20S β subunits were decreased. Also, immunohistochemical analysis of lumbar spinal cord revealed that similar to previously reported depletion in structural α subunits (Figure 11), β3 and β5 subunits (Figure 17) of the 20S core are decreased in lumbar motor neurons with disease progression.

During an immune and inflammatory response, cytokines such as IFN-γ induce up-regulation in the level of the inducible subunits, β5i, β2i and β1i, to replace the corresponding constitutive subunits, β5, β2, β1. The resulting "immunoproteasome" and are very efficient in generating several antigenic peptides (Rivett et al., 2001). We measured the levels of β5i in

lumbar spinal cord to determine if this occurs early in disease pathogenesis.of $SOD1^{G93A}$ transgenic mice. No changes in β5i were measured in the lumbar region of spinal cord of P45 and P75 $SOD1^{G93A}$ transgenic mice. These results strongly suggest that the impairment of proteasome activity and decreases in levels of 20S β subunits are very early events in fALS pathogenesis and occur prior to induction of an inflammatory response due to microglial activation (Figure 4). Also, no changes were measured in the levels of subunit S6b of the 19S regulatory particles, nor in 11Sα of the regulatory subunit of the immunoproteasome in the lumbar spinal cord of P45 and P75 $SOD1^{G93A}$ transgenic mice (Figure 15). These results demonstrate that regulatory particles and inducible subunits of the proteasome might not be involved in the early stages of fALS disease pathogenesis.

Levels of proteasomal subunit proteins were measured in lumbar spinal cord tissue of symptomatic $SOD1^{G93A}$ transgenic mice. As previously reported, an increase in the level of the inducible subunit, β5i protein levels occurs at this stage (Cheroni et al., 2005; Puttaparthi and Elliott, 2005) and an increase in the regulatory particle of the immunoproteasome, 11Sα, was associated with a decrease of subunit S6b of the 19S regulatory particle. These data suggest a fundamental change in proteasomal composition in the lumbar region of the spinal cord of $SOD1^{G93A}$ transgenic mice due to the glial response and the pronounced loss of motor neurons.

To determine whether this decrease in protein levels is due to transcriptional defects, rt-PCR was performed in laser captured motor neurons from various regions of the spinal cord. Surprisingly, the decrease in protein levels did not correspond to decreases in mRNA transcripts of any of these 20S core proteasomal subunits. These results confirm that the changes in protein levels of proteasomal subunits in the lumbar spinal cord and motor neurons are not due to transcriptional defects, but to possible defects in the assembly of proteasome complex and/or post-translational modifications of individual subunits. However, an increase

in certain subunits in spinal cord tissue of $SOD1^{G93A}$ transgenic mice that was not seen in motor neurons suggests that this increase might occur solely in non-neuronal cells. Similarly to protein chaperones (Batulan et al. 2003), non-neuronal cells, unlike motor neurons, might be able to up-regulate proteasomal subunits when the proteasome activity is inhibited.

Analysis of the composition of 20S/26S proteasomes by native gel electrophoresis demonstrated that in lumbar spinal cord tissue of $SOD1^{G93A}$ transgenic mice these complexes show unchanged immunoreactivity for α subunits, but decreased immunolabelling for β3 and β5 subunits. These results suggest complex changes in the structure of the proteasome in $SOD1^{G93A}$ transgenic mice during the course of the disease. The possibility that the constitutive β5 subunit was replaced in the proteasomal complexes with β5i was excluded since the levels of β5i did not increase as measured by Western blotting (Figure 13 and 14). Also, normal levels of β subunits were present as monomers which exclude the possibility that these subunits are rate-limiting in lumbar spinal cord tissue as was suggested by cell lines studies (Chondrogianni et al., 2003).

Another possibility is post-translational modifications to 20S β subunits that impair recognition by the antibodies and/or affect incorporation into proteasomal complexes. A major post-translational modification of 20S subunits that affects proteasomal activity and complex formation is HNE-modifications of certain amino-acids (Farout et al., 2006). An antibody immunoreactive to HNE adducts was obtained from Dr. Luke Szweda (University of Oklahoma Health Sciences Center). Immunoprecipitation and Western blotting of lumbar spinal cord of $SOD1^{G93A}$ transgenic mice were performed, but no HNE-modified proteasomal subunits were detected in these Western blots (data not shown). Semi-purification of proteasome complexes by gradient centrifugation and analysis of the subunits by 2D gel electrophoresis is required to detect HNE-modified proteasomal subunits *in vivo* (Dr. Szweda, personal communication).

Another option was to immunoprecipitate intact proteasomal complexes and use mass-spectrometry analysis to determine protein composition and post-translational modifications of proteasomal subunits. In our preliminary experiments, the only commercially available antibody known to immunoprecipitate intact proteasomes that recognizes α subunit of the 20S core, immunoprecipitated the subunit, but not the whole 20S complex as determined by co-labelling with antibodies to β subunits. Finally, spots from the second dimension of blue native gels that correspond to 20S proteasomal bands in murine tissue were excised and their identity was analyzed by mass spectrometry. However, the signal was very low only 20S α subunits were recognized and the sequence coverage was minimal (2-7%).

Due to technical limitations in proteasomal subunit purification and characterization, we could not study the post-translational modification of subunits in affected tissues of $SOD1^{G93A}$ transgenic mice. An alternative possibility to explain a reduction proteasomal activity without a decrease in the expression of structural α subunits is alteration in proteasome complex assembly. However, from our experiments using native gel electrophoresis, 20S and 26S proteasomes from lumbar spinal cord of $SOD1^{G93A}$ transgenic mice run as intact complexes similar to complexes from spinal cord tissue of non-transgenic littermates. Also, no increase in free subunits or intermediate proteasomal complexes were observed implying there is no obvious structural changes in proteasome complex formation. Further studies are required to resolve which mechanisms are involved in proteasome activity decrease observed in affected tissue of mutant SOD1 transgenic mice. What remains of essence from these results is that impairment of the proteasome activity and a corresponding decrease of the β subunits of the 20S proteasome occur early in disease pathogenesis and can contribute to fALS motor neuronal pathology in the transgenic mouse model.

Studies of neuronal cell lines have demonstrated that mutant SOD1 aggregates associate with subunits of the 20S core and this association leads to proteasome overload and

progressive inhibition of its activity (Matsumoto et al., 2005). To determine if this phenomenon occurs *in vivo*, an antibody specific to human SOD1 (SOD100) was used to test whether mutant SOD1 associates with the proteasome complex (Figure 21A) and whether SOD1 immunodepletion of SOD1 relieves proteasome impairment measured in lumbar spinal cord of $SOD1^{G93A}$ transgenic mice. This antibody can recognize high-molecular weight, SDS-insoluble, SOD1 species that accumulate in spinal cord during the course of the disease (Figure 6) and oxidatively modified mutant SOD1 (Agar, Kabashi et al. manuscript in preparation). However, immunodepletion of soluble SOD1 from the lumbar spinal cord did not relieve the proteasome impairment (Figure 21B). Also, mutant SOD1, including SDS-insoluble SOD1 species did not migrate with the 20S proteasome complex as seen in the 2^{nd} dimension of Blue Native gels (Figure 21A). These experiments argue against direct association and choking of the proteasome complex by mutant SOD1 (Bennett et al., 2005). However, these results do not exclude the possibility that post-translationally modified forms of mutant SOD1 that are not recognized by the antibody might lead directly to proteasome impairment. Interestingly, this antibody does not recognize the cleaved SOD1 peptides from the 20S proteasome that have been proposed to be associated with toxicity (Di Noto et al., 2005), and it does not label the inclusion bodies formed when various forms of mutant SOD1 are injected in motor neurons (Durham et al., 1997). Full biophysical characterization of mutant and wild type SOD1 proteins from tissues will be required to determine which states occur under physiological conditions and correlate with disease markers. Generation of reliable antibodies to recognize these modified forms of SOD1 for immunoprecipitation and mass spectrometry characterization of these peptides are also required.

In conclusion, these studies identify two events occurring simultaneously in lumbar spinal cord tissue during disease progression in $SOD1^{G93A}$ transgenic mice; a focal impairment of proteasome activity associated with decreased levels of 20S β subunits early in

disease pathogenesis and a progressive decrease of 20S α and β subunits in lumbar motor neurons that peaks at symptomatic stages. Further research is required to determine what causes these changes in enzymatic activity and protein levels of the proteasomal machinery and whether the phenomena described here occur in affected tissues from sporadic and fALS patients as well as in other animal models of fALS1.

Figure 13

The decrease in constitutive 20S core β subunits initiates at P45 in SOD1^{G93A} transgenic mice and is not associated with changes in inducible β subunits

Expression of specific proteasome subunits measured by SDS/PAGE immunoblotting of soluble extracts of lumbar spinal cord (10 μg per lane) from four groups of mice: SOD1^{G93A} transgenic mice, SOD1^{WT} transgenic mice and their age-matched non-transgenic littermates (GLm and WLm). Spinal cord tissue extracts from two litters of P45 SOD1^{G93A} transgenic mice and one litter of SOD1^{WT} transgenic mice were analyzed in duplicate.

The bands on exposed blots from representative Western blots in (A) were normalized to the levels of actin, which was used as loading control. A significant decrease of two β subunits, β3 (structural subunit) and β5 (harbouring the chymotrypsin-like activity) were measured solely in lumbar spinal cord extracts of SOD1^{G93A} transgenic mice. No changes were measured in 20S α subunits or in the inducible β subunit (β5i) (* $p<0.05$).

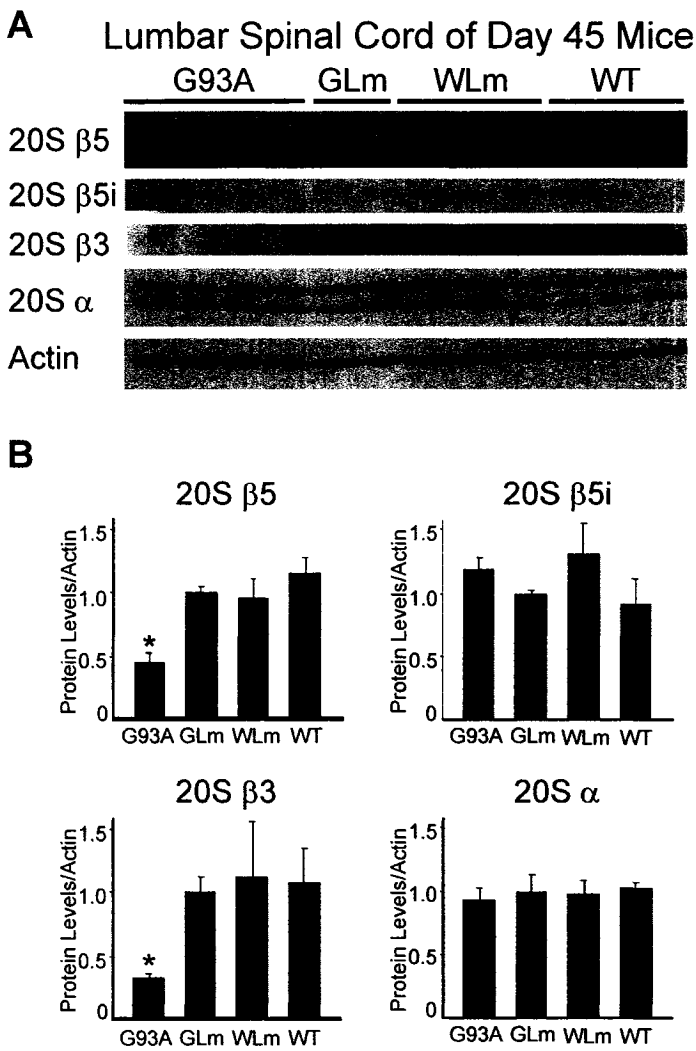

A
Lumbar Spinal Cord of Day 45 Mice
G93A
GLm
WLm
WT
20S β5
20S β5i
20S β3
20S α
Actin
B
20S β5
20S β5i
20S β3
20S α
Protein Levels/Actin
1.5
1.0
0.5
0
*
G93A GLm WLm WT

Figure 13

Figure 14

The decrease in β subunits is accentuated in P75 SOD1^{G93A} transgenic mice, whereas the expression of inducible β and α subunits of the 20S core were not affected

A. Western blots of soluble spinal cord extracts (10 μg per lane) separated by SDS-PAGE probed with antibodies specific to various proteasomal subunits. Four groups of mice were analyzed: SOD1^{G93A} transgenic mice, SOD1^{WT} transgenic mice and their age-matched non-transgenic littermates (GLm and WLm). Spinal cord tissue extracts from two litters of P75 SOD1^{G93A} transgenic mice and one litter of SOD1^{WT} transgenic mice were used. A significant decrease of two β subunits, β3 (structural subunit) and β5 (harbouring the chymotrypsin-like activity) were measured solely in lumbar spinal cord extracts of SOD1^{G93A} transgenic mice. No changes were measured in other proteasomal subunits, including 20S α subunits, 19S5a and 11Sα. Also, no concurrent increase in the inducible β subunit (β5i) was observed.

B. Exposed films from Western blots using spinal cord tissue extracts from two litters of P45 SOD1^{G93A} transgenic mice and one litter of P75 SOD1^{WT} transgenic mice were quantified using the ScionData program. All results shown in (A) were repeated twice in different Western blots and normalized to actin levels, which were used as loading control. Quantification of the exposed blots in (A) reveals an impairment of 20S β subunits that is not associated with changes in other proteasomal subunits. The decrease of β5 and β3 with no increase in the inducible β5i subunit of the 20S was repeated in at least three litters of SOD1^{G93A} transgenic mice and age-matched non-transgenic littermates (* $p<0.05$).

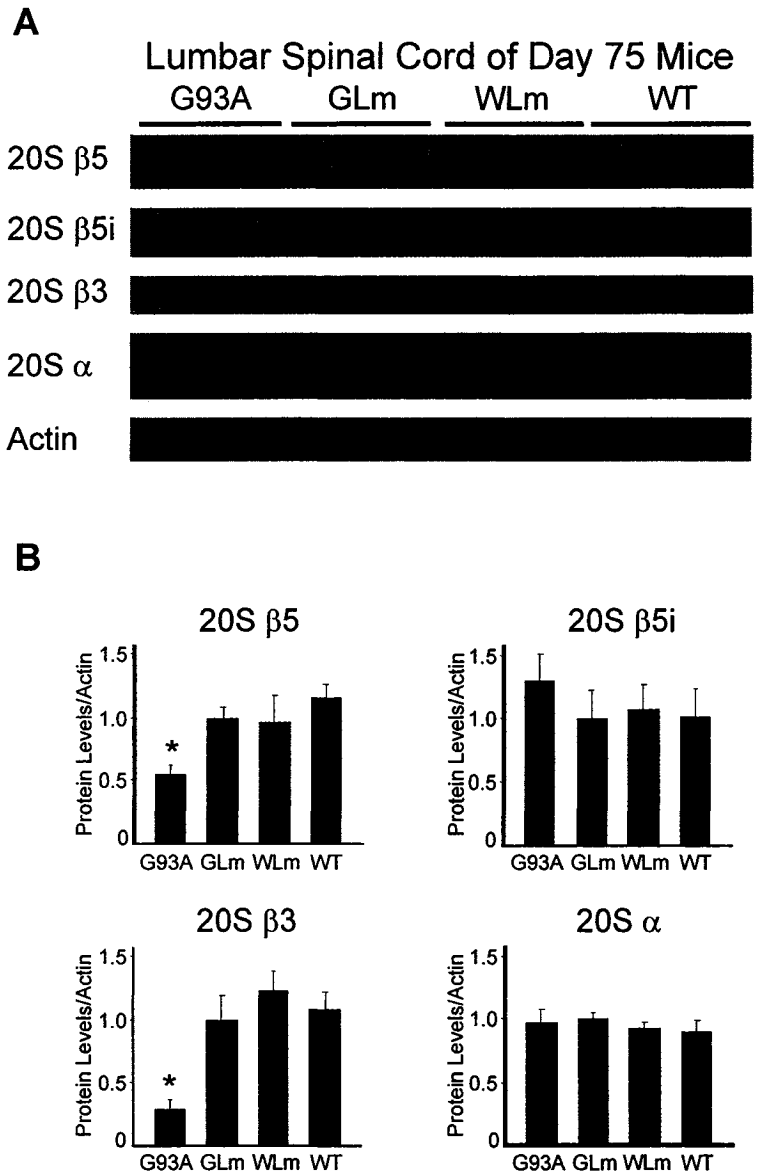

A
Lumbar Spinal Cord of Day 75 Mice
G93A
GLm
WLm
WT
20S β5
20S β5i
20S β3
20S α
Actin
B
20S β5
20S β5i
20S β3
20S α
Protein Levels/Actin
1.5
1.0
0.5
0
*
G93A GLm WLm WT

Figure 14

Figure 15

No changes in 20S subunits in thoracic spinal cord of pre-symptomatic SOD1^{G93A} transgenic mice. Also, two subunits of the regulatory particle (19S and 11S) of the proteasome were not affected in the lumbar spinal cord in the pre-symptomatic stages

A. Thoracic spinal cord tissue obtained from P45 and P75 SOD1^{G93A} transgenic mice and age-matched non-transgenic littermates were immunobloted for 20S β (β5, β5i, β3) and for α subunits of the 20S core of the proteasome. No change was detected (panel B) in the expression of any of these subunits. This confirms that the pre-symptomatic changes in 20S constitutive β subunits is focal to the lumbar region and corresponds to specific impairment of proteasome activity in this region.

B. Western blots from Figure 12 and 13 were reprobed with antibodies to subunit S6b of the 19S and 11Sα regulatory particles of the proteasome. We determined no pre-symptomatic decrease in their levels (panel D) suggesting that the expression of the regulatory particles is not altered at the early stages of ALS pathogenesis (* $p<0.05$).

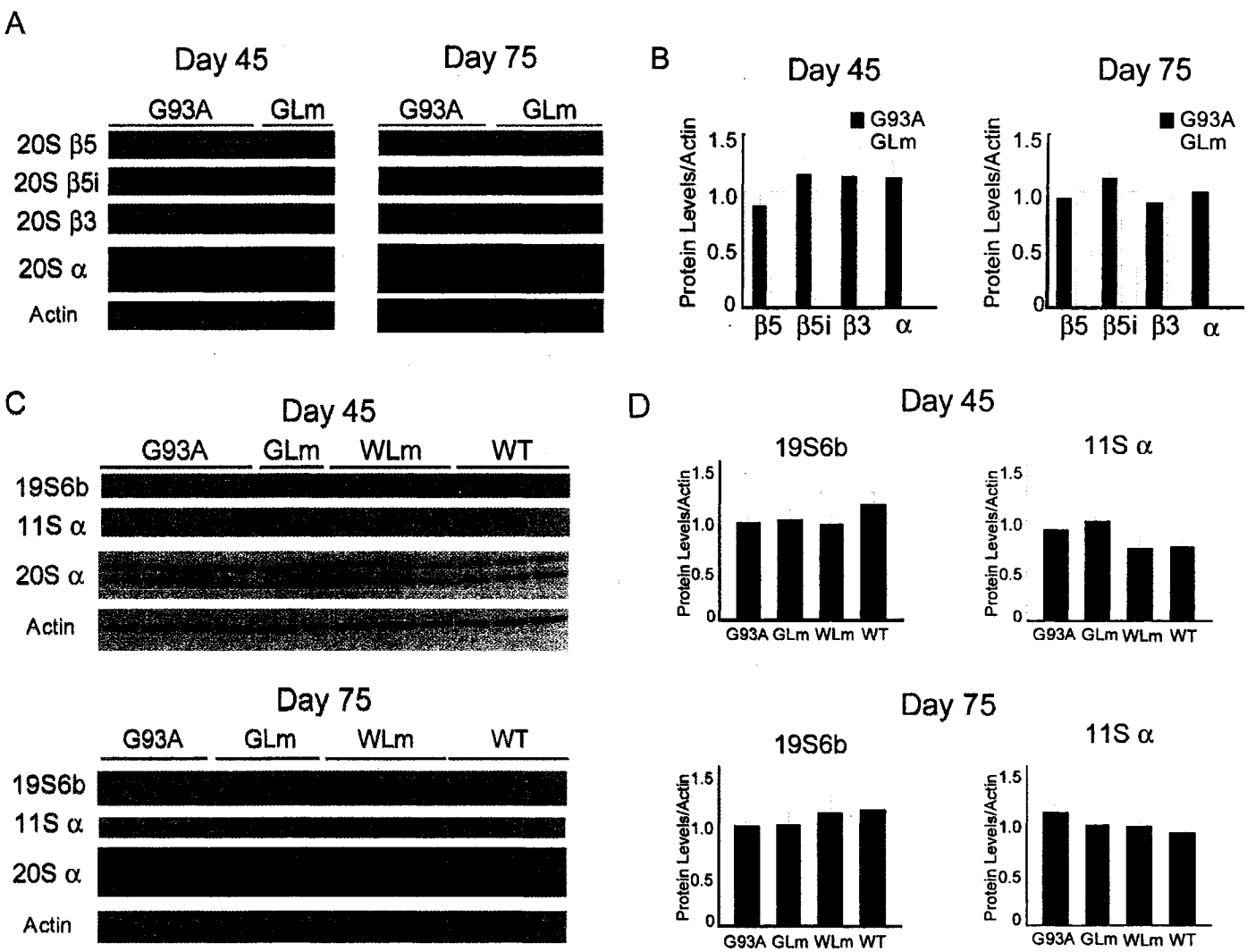

A
Day 45
Day 75
G93A
GLm
G93A
GLm
20S β5
20S β5i
20S β3
20S α
Actin
B
Day 45
Day 75
G93A
GLm
Protein Levels/Actin
1.5
1.0
0.5
0
β5 β5i β3 α
C
Day 45
G93A GLm WLm WT
19S6b
11S α
20S α
Actin
Day 75
G93A GLm WLm WT
19S6b
11S α
20S α
Actin
D
Day 45
19S6b
11S α
Protein Levels/Actin
G93A GLm WLm WT
Day 75
19S6b
11S α
Protein Levels/Actin
G93A GLm WLm WT

Figure 15

Figure 16

The decrease in 20S core constitutive β subunits in symptomatic SOD1^{G93A} transgenic mice is associated with an increase in levels of the inducible β subunits and the regulatory particle (11S) of the immunoproteasome

A. Western blots of soluble spinal cord extracts (10 μg per lane) separated by SDS-PAGE probed with antibodies specific to various proteasomal subunits. Four groups of mice were analyzed: symptomatic SOD1^{G93A} transgenic mice and their age-matched non-transgenic littermates (GLm approximately 120-130 day old). Spinal cord tissue extracts from two litters of symptomatic SOD1^{G93A} transgenic mice were used. Increased protein levels of 20S β5i and the regulatory subunit of the immunoproteasome, 11Sα, confirm that there is an elevation of the immunoproteasome expression in lumbar spinal cord of symptomatic SOD1^{G93A} transgenic mice due to the glial inflammatory response. Levels of β5 and 19S6b were found to be decreased at this stage reflecting large changes in spinal cord proteasome composition at this late stage of the disease.

B. Western blots were quantified revealing significant increases in protein levels of 20S β5i and 11S as well as significant decreases in 20S β5 and 19S6b, whereas levels of 20S α subunits were not changed. Quantified levels were normalized to actin which was used as a loading control (* $p<0.05$).

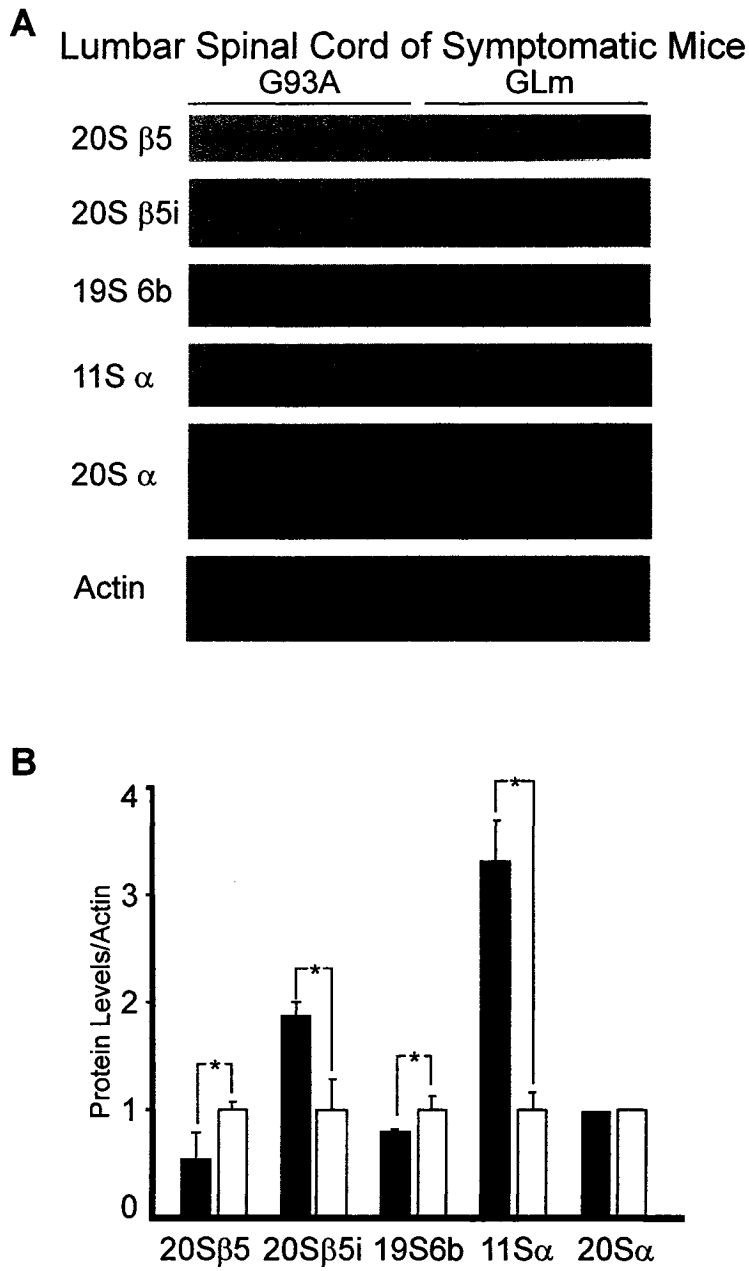

A
Lumbar Spinal Cord of Symptomatic Mice
G93A
GLm
20S β5
20S β5i
19S 6b
11S α
20S α
Actin
B
Protein Levels/Actin
0
1
2
3
4
*
20Sβ5
20Sβ5i
19S6b
11Sα
20Sα

Figure 16

Figure 17

Expression of constitutive 20S β subunits in lumbar spinal motor neurons of symptomatic SOD1^{G93A} transgenic mice

Expression of (A) β3 and (B) β5 subunits in lumbar motor neurons of SOD1^{G93A} transgenic mice with disease progression determined by immunolabeling 20 μm spinal cord sections. Motor neurons express high levels of constitutive β as well as α subunits of the 20S core of the proteasome (see Figure 10). A progressive decrease in β5 and β3 immunolabeling was observed in motor neurons as the disease progressed, but not in age-matched non transgenic LM.

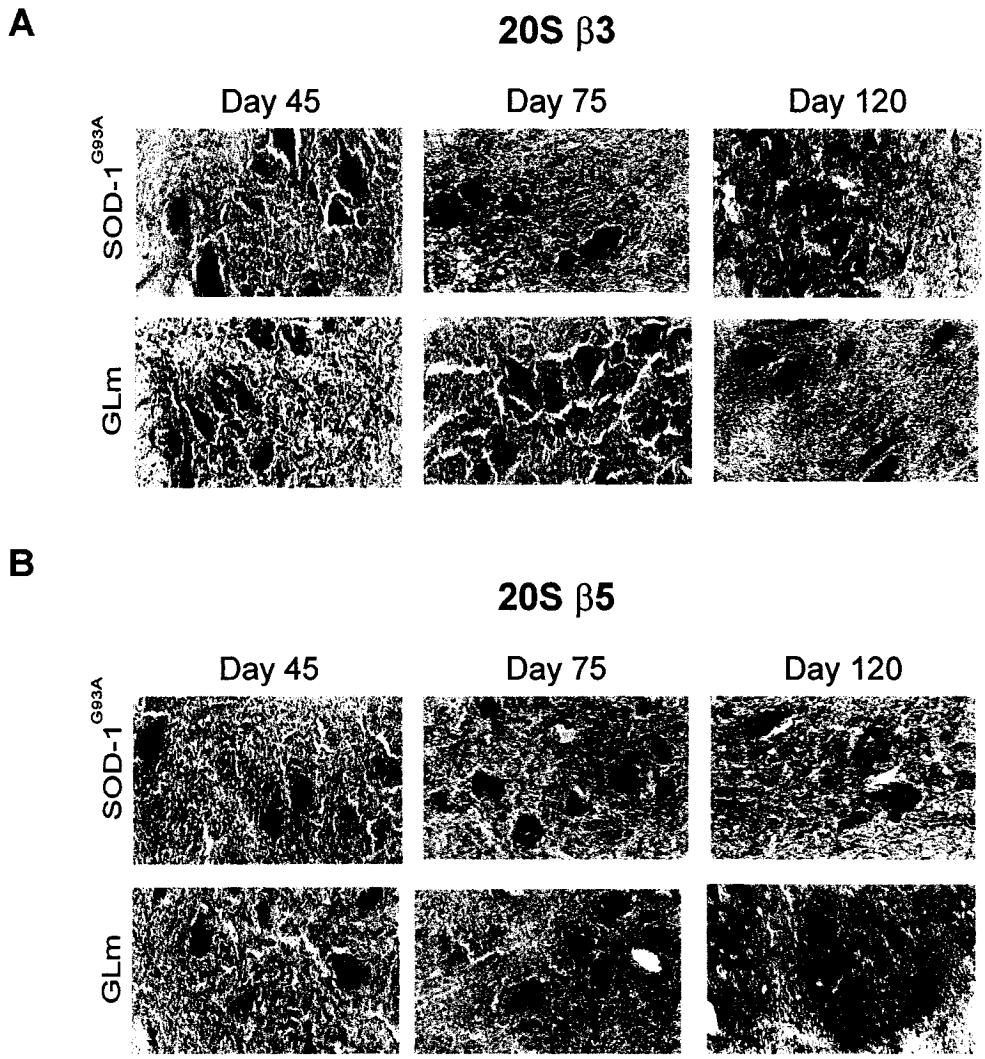

A
20S β3
Day 45
Day 75
Day 120
SOD-1G93A
GLm
B
20S β5
Day 45
Day 75
Day 120
SOD-1G93A
GLm

Figure 17

Figure 18

No reduction in expression of proteasome subunit mRNAs in the spinal cord of mutant SOD1 transgenic mice

Shown is expression of mRNAs encoding murine β3, β5, β5i and α7 proteasome subunits relative to GAPDH as a control housekeeping gene. rt-PCR of whole tissue extracts of lumbar, thoracic and cervical spinal cord was performed using specific primers listed in Table 4. No decrease in levels of 20S subunit mRNAs was measured. Instead, a significant increase in β5 mRNA levels in lumbar and cervical spinal cord of $SOD1^{G93A}$ transgenic mice was determined, as well as an increase of β5i in the thoracic and cervical sections of the spinal cord. Also, mRNA levels of the 20S α7 subunit were increased in thoracic spinal cord extracts from $SOD1^{G93A}$ transgenic mice (* $p<0.05$).

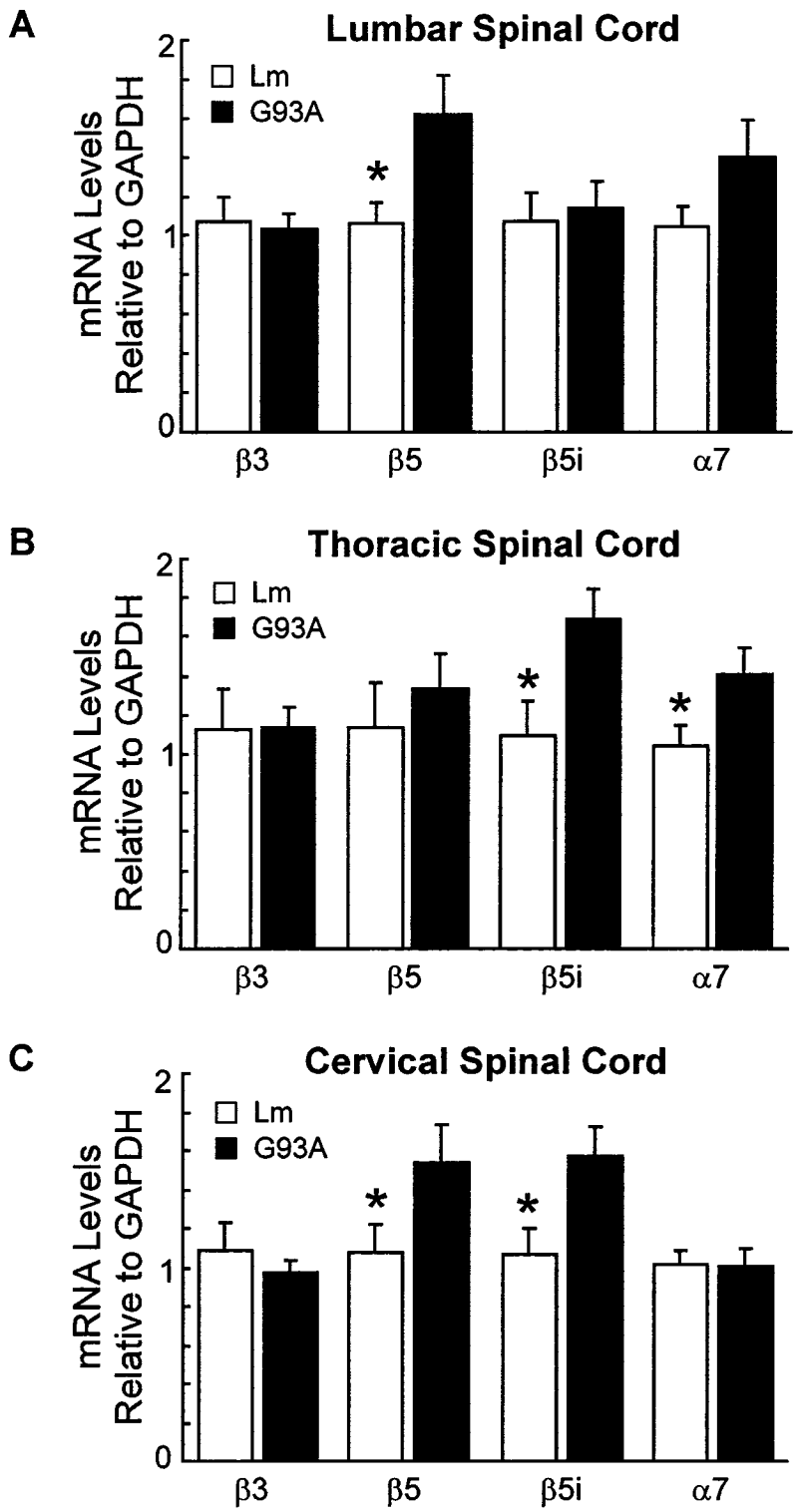

A
Lumbar Spinal Cord
mRNA Levels Relative to GAPDH
Lm
G93A
2
1
0
*
β3
β5
β5i
α7
B
Thoracic Spinal Cord
mRNA Levels Relative to GAPDH
Lm
G93A
2
1
0
*
*
β3
β5
β5i
α7
C
Cervical Spinal Cord
mRNA Levels Relative to GAPDH
Lm
G93A
2
1
0
*
*
β3
β5
β5i
α7

Figure 18

Figure 19

Normal levels of 20S subunit mRNAs despite decrease in 20S core protein levels in lumbar motor neurons of symptomatic SOD1^{G93A} transgenic mice

To analyze expression of mRNAs encoding proteasome subunits specifically in motor neurons, motor neurons were removed by laser microdissection from sections of lumbar, thoracic and cervical regions of spinal cord from P100 SOD1^{G93A} transgenic mice and non-transgenic LM. Panel A shows section before (upper) and after (lower) laser capture of a motor neuron from an SOD1^{G93A} transgenic mouse.

B. Immunolabeling of sections of lumbar spinal cord with antibody to proteasome core shows reduction in labeling of 20Sα subunits in SOD1^{G93A} transgenic mice relative to LM, but maintained expression of the control housekeeping protein, GAPDH.

C. Quantitation of reverse transcribed mRNA in approximately 20 pooled motor neurons per primer by rt-PCR revealed no significant difference in levels of 20Sα7, β3 or β5 subunits from lumbar, thoracic and cervical motor neurons of SOD1^{G93A} transgenic mice or LM. β5i mRNA was significantly increased possibly due to the immune response that is a prominent feature at this stage of disease (* $p<0.05$).

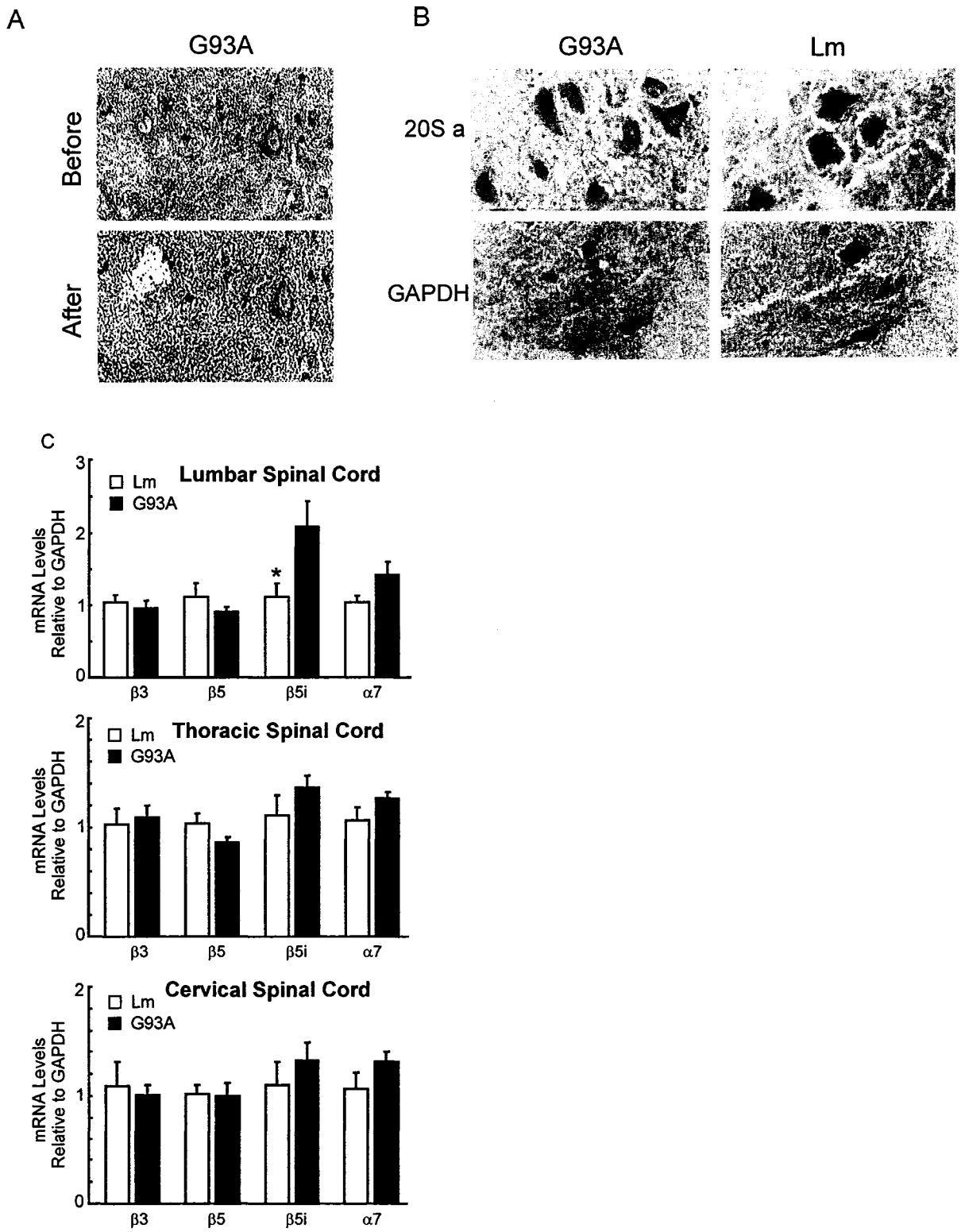

A
G93A
Before
After
B
G93A
Lm
20S a
GAPDH
C
Lumbar Spinal Cord
Lm
G93A
mRNA Levels Relative to GAPDH
β3
β5
β5i
α7
Thoracic Spinal Cord
Cervical Spinal Cord

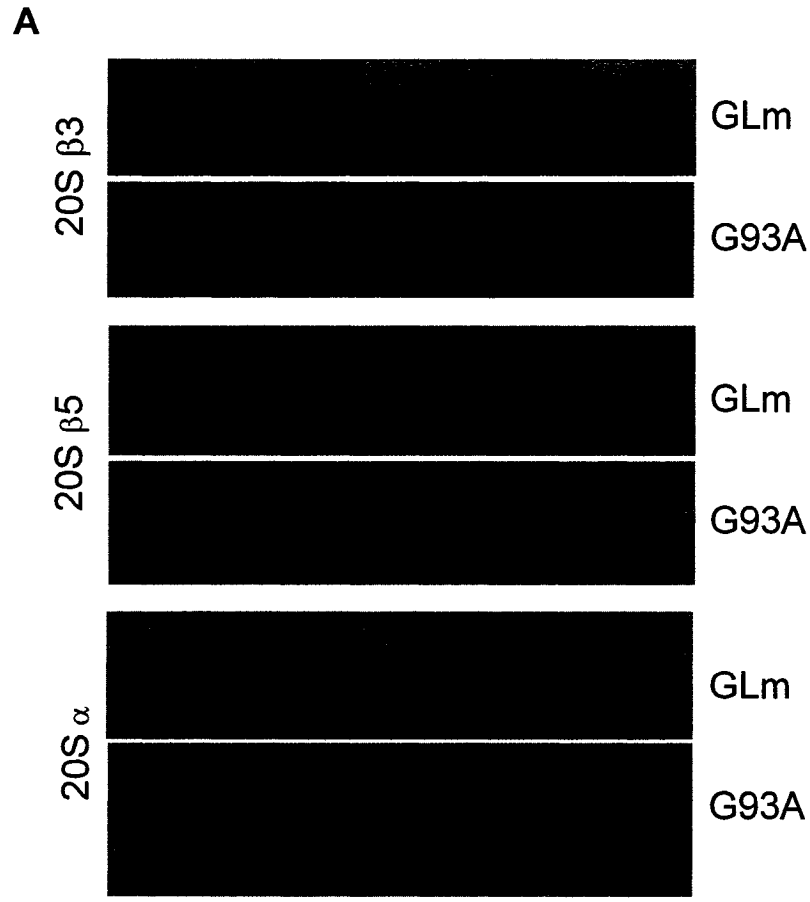
Figure 19

Figure 20

The assembly of 20S and 26S proteasome complexes is not impaired whereas incorporation of β subunits is affected in the lumbar spinal cord of SOD1^{G93A} transgenic mice

A. 2-D Blue native/SDS-PAGE was used to separate protein complexes (including monomeric α and β subunits of the 20S core, immature precursors, and assembled proteasome complexes) in the first dimension, then analyze subunit composition of complexes in the second, denaturing dimension. By this method, most of the proteasomal complexes were 20S because they were labelled by 20S core antibodies, but not by antibodies to 19S regulatory particle (data not shown). Extracts of lumbar spinal cord from P75 SOD1^{G93A} transgenic mice and nontransgenic LM were analyzed. Second dimension gels were transferred to nitrocellulose and labelled with antibodies to 20Sα, β3 and β5 proteasome subunits. Antibodies labelled proteins of the same high molecular weight mobility representing 20S proteasome (major bands on left side of blots), with very few monomeric subunits being detected (small spots on far right of blots). Immunolabeling of β5 and β3 subunits in the assembled 20S core was reduced in lumbar spinal cord of P75 SOD1^{G93A} transgenic mice relative to LM, without concomitant increase in species with lower molecular weight.

B. To maintain integrity of 26S complexes, a protocol modified from that developed by Finley and colleagues was utilized. A complex approximately 2.5 MDa in size was observed upon probing blots of lumbar or thoracic spinal cord with antibodies to α, and β 20S subunits as well as the S5a subunit of the 19S regulatory particle. As with 20S complexes shown in (A), β5 and β3 immunolabeling was decreased in in the assembled 26S complexes of lumbar spinal cord of P75 SOD1^{G93A} transgenic mice without any change in labeling of 20S α subunits.


A
20S β3
GLm
G93A
20S β5
GLm
G93A
20S α
GLm
G93A

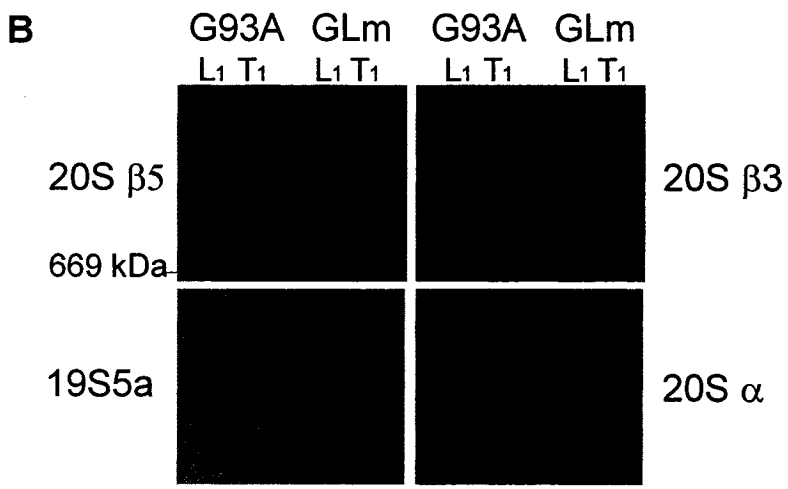

B
G93A
L1 T1
GLm
L1 T1
G93A
L1 T1
GLm
L1 T1
20S β5
669 kDa
19S5a
20S β3
20S α

Figure 20

Figure 21

Mutant SOD1 was not associated with proteasome complexes in lumbar spinal cord from SOD1^{G93A} transgenic mice

A. Western blots prepared from 2-D Blue native/SDS-PAGE of lumbar spinal cord extracts from P75 SOD1^{G93A} transgenic mice were probed with antibodies to 20Sα and SOD1 (SOD100; Stressgen) to determine any association of mutant SOD1 with proteasome complexes. No co-migration of mutant SOD1 with the proteasome was observed. Overexposure of the blots revealed high molecular weight SOD1 species in the lumbar region of SOD1^{G93A} transgenic mice spinal cord in the low molecular weight region of the gel, not with proteasomes.

B. SOD1 was immunodepleted (IMD) from lumbar spinal cord homogenates from three individual mice using an antibody that preferentially binds human SOD1. Left-most bands in exposed Western blot using the SOD100 antibody represent pre-immunodepleted samples from lumbar spinal cords of three SOD1^{G93A} transgenic mice (L1, L2, L3). To make quantitation possible, immunodepleted samples were run at ten-times the concentration of pre-IMD samples and are shown on the right of each pre-immunodepleted sample. According to quantitation of band densities by Scion Image, approximately 93% of total human SOD1 was immunodepleted from the lumbar spinal cord of SOD1^{G93A} transgenic mice. Immunodepleting mutant SOD1 from lumbar spinal cord extract did not affect proteasome activities as measured by hydrolysis of peptide substrates.

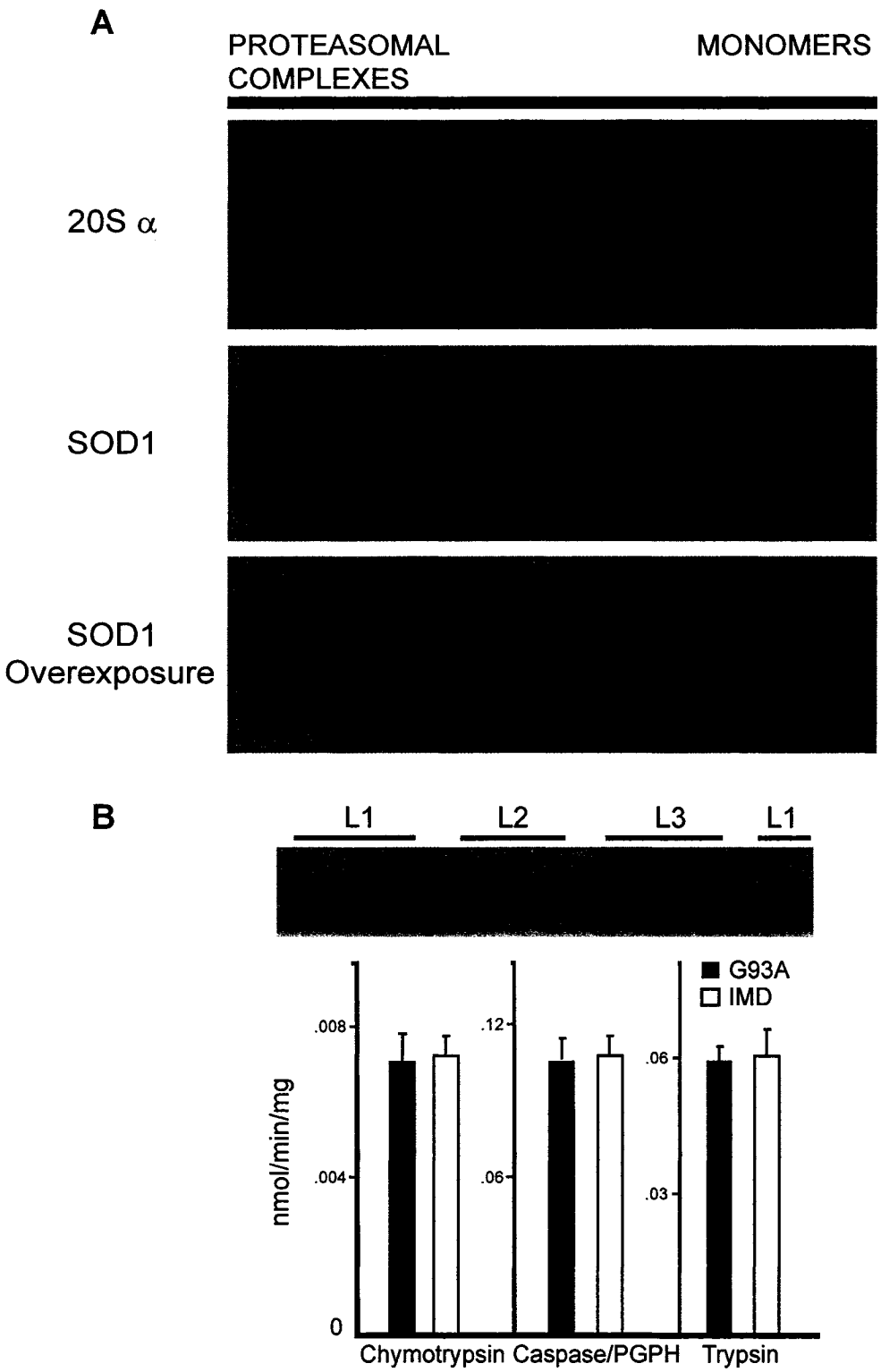

A
PROTEASOMAL COMPLEXES
MONOMERS
20S α
SOD1
SOD1 Overexposure
B
L1
L2
L3
L1
G93A
IMD
nmol/min/mg
.008
.004
0
.12
.06
.06
.03
Chymotrypsin
Caspase/PGPH
Trypsin

Figure 21

Chapter 6 Role of the ubiquitin-proteasome pathway in sporadic ALS

Experiments in this chapter were conducted in order to determine if protein levels and activities of various proteasomal proteins and complexes are altered in autopsy tissue from sporadic ALS patients (Specific Aim 3). Three main activities of the proteasome were determined in dorsal and ventral sections of the thoracic spinal cord (an affected region) and less affected CNS tissue (cerebellum) obtained post-mortem from sporadic ALS patients and control cases. Also, the levels of α and β subunits of the 20S core, and representative subunits of the regulatory particles (19S and 11S) of the proteasome were assessed. As in the fALS mouse model, reduction in all three proteasomal activities was measured in thoracic spinal cord of ALS patients. This was accompanied by a decrease of the 20S catalytic subunit β5, but no significant changes in the expression of other proteasomal subunits were measured.

6.1 Evidence of the ubiquitin-proteasome pathway involvement in sporadic ALS

Studies in experimental models point to inadequacy of cellular stress responses and dysfunction of the proteasomal proteolytic pathway in the pathogenesis of familial ALS due to mutations in SOD1. Decreased specific proteasomal enzymatic activities in lumbar spinal cord of $SOD1^{G93A}$ transgenic mice early in pathogenesis was restricted to lumbar spinal cord tissue, the region eventually most affected by disease (refer to Chapter 4.2). This was compounded by altered expression of at least two β subunits in lumbar spinal cord as well as depletion of 20S core subunits in lumbar motor neurons later in the disease (see Chapter 4 and 5). It is important to determine the relevance of these findings in experimental models to pathogenesis of sporadic ALS patients since they account for about 90% of cases. The

working hypothesis was that compromise in handling of abnormal proteins is an early, common event in ALS pathogenesis.

Cytosolic protein inclusion bodies visible by light microscopy are common in sporadic (Leigh et al., 1988; Shibata et al., 2001) and fALS (Kato et al., 2000; Watanabe et al., 2001), mutant SOD1 transgenic mice (Dal Canto and Gurney, 1995; Bruijn et al., 1997) as well as primary cultured motor neurons (Durham et al., 1997), suggesting that protein misfolding and aggregation play an important role in ALS pathogenesis generally. Since these inclusions are often labeled positively by antibodies specific to ubiquitin, it was suggested that the ubiquitin-proteasome pathway is involved in degrading the proteins that are trapped in these inclusion bodies.

To evaluate the role of proteasome dysfunction in sporadic ALS, it was essential to measure proteasome activities, to evaluate the expression of its subunits, as well as to determine if any changes occurred globally or were restricted to tissues affected in this neurodegenerative disorder.

6.2 Feasibility of Assessing Levels and Activities of Proteasomes in Post-Mortem Tissue of ALS Patients

Since interpretation of biochemical measurements in postmortem, end-stage tissue can be challenging, it was important to determine levels and activities of the proteasome in banked autopsy tissue and to compare it to results in murine tissues using the same methodology as results presented in Chapter 4 and 5. Also, the stability of the proteasome complexes for at least 12 hours post-mortem was determined in murine tissue by measuring proteasome activities as well as protein levels of structural α subunits of the 20S core.

Experimental Approach and Results

6.2.1 Autopsy Time Does Not Have an Effect in Proteasome Activity and Levels

In order to determine if activity and levels of the proteasome change with post-mortem interval (refer to Table 3), a preliminary study was conducted in mice. Murine spinal cord tissue was frozen immediately after dissection or left at room temperature at various time point intervals up to 12 hours. Six non-transgenic mice with B6SJL background were used. Spinal cord was removed and cut in half and hemicords were placed in tubes and frozen immediately (0 hours), or left in the dark at room temperature and placed at -80°C 3, 6 and 12 hours after spinal cord removal. Liver tissue from these mice was also removed and immediately frozen. No changes in the chymotryptic activity of the proteasome were observed among all four test conditions (frozen right away, t = 0; frozen after 3, 6, and 12 hours left at room temperature) (Figure 22A). Western blot analysis showed that the postmortem interval did not affect levels or integrity of protein bands representing α structural subunits of the 20S core or actin (Figure 22B). It was concluded that post-autopsy intervals up to 12 hours do not have a significant impact in proteasome activity and levels, and therefore conducting these experiments using postmortem human tissue was feasible and meaningful.

6.1.2 Activity and Levels of Proteasome in Frozen Human Spinal Cord is Comparable to Murine Tissue

The next step in this series of control experiments was to measure all three proteasomal activities in human autopsy tissue maintained frozen at -80°C and compare these activities and levels of proteasome to murine tissue. The three proteolytic activities of the proteasome, namely chymotrypsin-like (Figure 23A), caspase-like (Figure 23B) and trypsin-like (Figure 23C) activities, and levels of 20Sα subunits (Fig. 23D) were measured in spinal cord tissue from a control case (Fig. 23A,B,C; bars in right-hand side) and from liver tissue of a nontransgenic mouse (Fig. 23A,B,C; bar in left-hand side). The data was normalized to

murine spinal cord tissue from previous experiments (Fig. 23A,B,C; right panels). Also, protein levels of α structural subunits of the 20S core in frozen human and murine spinal cord samples were determined. The activities and levels of the proteasome in frozen human tissue were remarkably similar to frozen mouse tissue (Figure 23).

6.3 Impairment of the Ubiquitin-Proteasome Pathway in the Spinal Cord of Sporadic ALS Patients

Having established that meaningful measurements of proteasome composition and activity are feasible in frozen human autopsy tissue, we proceeded with experiments to compare these measures in tissues from sporadic ALS patients and non-neurological controls and to conduct an immunohistochemical analysis of expression of proteasome subunits. Frozen tissue (spinal cord and cerebellum) as well as paraffin-embedded spinal cord sections from the same region were obtained from the tissue bank of Dr. Michael Strong (Robarts Research Institute, London, Ontario).

Experimental Approach and Results

6.3.1 Focal Dysfunction of Proteasome Activity in Spinal Cord of Sporadic ALS Patients

20S/26S proteasomal activities were measured in ventral as well as dorsal section of the spinal cord, (Fig. 24A,B,C left panels) tissue extracts obtained from five sporadic ALS patients (thoracic region of the spinal cord), thoracic region of two control cases and cervical spinal cord from two other controls (cervical). Proteasome activities were also measured in cerebellar tissue from the same individuals (Fig. 24A,B,C right panels). Assays were based on hydrolysis of fluorogenic substrates specific for each of the chymotrypsin-like, caspase-like and trypsin-like activities of the proteasome. Chymotrypsin-like activity of the proteasome, calculated as nmol of AMC/min/mg, was significantly impaired in the thoracic spinal cords,

activity in the ventral and dorsal regions of ALS samples being 60% and 65% of the activity in control cases, respectively (Figure 24A). Also the other two major activities of the proteasome were significantly reduced, with caspase- (Fig. 23B) and trypsin-like (Figure 24C) activities in spinal cord of ALS tissue being 54%, and 65%, in the ventral region and 68% and 46% in the dorsal region respectively, of the activities measured in control cases. No significant reduction in any of the three proteasomal activities were measured in cerebellar tissue of sporadic ALS patients (Fig. 24A,C,D left panel), tissues previously shown to exhibit limited pathological changes (Tandan and Bradley, 1985). In summary, a focal reduction of proteasome activity was measured within the thoracic region of the spinal cord of sporadic ALS patients.

6.3.2 Decreased Levels of β5 Catalytic Subunit Corresponds to Proteasome Impairment in Tissues Affected by Sporadic ALS

Focal reduction of the proteasome activity, normalized to levels of 20Sα structural subunits, corresponded to decreased levels of 20S structural β3 and functional β5 subunits in mutant SOD1 transgenic mice. To determine if the levels of 20S subunits and subunits of the regulatory particles, 19S and 11S are affected in sporadic ALS, SDS/PAGE and Western blotting were conducted using extracts of spinal cord and cerebellum from the five ALS and four control cases. The amount of catalytic 20S β5 subunit was decreased in thoracic spinal cord, but not cerebellum of ALS samples, but was not due to an increase in the expression of the inducible subunit, β5i (Figure 25A). Also, a decrease measured in levels of S5a subunit of the 19S was not associated with an increase in the levels of the regulatory particle of the immunoproteasome, 11S α (Figure 25B). The decreases of 20S β5 and 19S5a were specific to spinal cord tissue since no changes were observed in the cerebellar tissue extracts (Figure

25B). Unlike the fALS mouse, no changes in the structural β3 subunit of the 20S core were observed.

6.3.3 Further Evidence of Impairment of the ubiquitin-proteasome pathway in Spinal Cord Tissue of ALS Patients

As previously discussed, proteasome inhibition in rat substantia nigra leads to a rapid up-regulation of mRNA and protein levels of Hsp70, but not Hsp40 (Ahn and Jeon, 2006). In a previous study from our laboratory, no upregulation of HSP70 was observed in motor neurons of ALS spinal cord, except for labeling of occasional inclusion bodies (Batulan et al., 2003). However, when levels of HSP70 and HSP40 were examined in the present study by Western blotting of tissue extracts from sporadic ALS and control cases, Hsp70 levels were increased in thoracic spinal cord of ALS patients, whereas levels of Hsp40 were not affected. This is another similarity between the fALS mouse model and sporadic ALS and is evidence of an increased load of misfolded proteins, which could accumulate if proteasomal degradation is inadequate.

6.4 Discussion

Since most inclusion bodies from sporadic and fALS patients were immunopositive for ubiquitin, a dysfunction of the ubiquitin-proteasome pathway was hypothesized as an essential factor for protein aggregation into inclusion bodies in ALS pathogenesis. However, one antibody raised against the 20S core failed to label these ubiquitin-positive inclusion bodies in motor neurons and astrocytes from sporadic and fALS cases (Watanabe et al., 2001). Recently, a paper published while this thesis was in preparation, and using another 20S proteasome antibody, reported an increase in proteasome immunolabeling in motor neurons with inclusion bodies suggesting an up-regulation of the proteasome complex in sporadic

ALS (Mendonca et al., 2006). This study is in discordance with results from two independent studies in $SOD1^{G93A}$ transgenic mice where a decrease of proteasomal subunit levels was observed in motor neurons as disease progressed (Figure 11) (Kabashi et al., 2004). In fact, Cheroni et al. illustrated by double-label immunocytochemistry that 20S core levels were lowest in motor neurons with ubiquitin-positive inclusion bodies (Cheroni et al., 2005). Antibody non-specificity as well as epitope masking in human tissue might have contributed to this discrepancy. Studies using antibodies directed against specific subunits of 20S, 19S and 11S as well as ubiquitinated proteins are needed to determine levels of components of ubiquitin-proteasome pathway in affected tissue from ALS patients. Various protein chaperones have been found in protein aggregates from $SOD1^{G93A}$ and $SOD1^{G85R}$ transgenic mice, including moderate expression of Hsp40, Hsp60, Hsp70, Hsp90 as well as strong expression of Hsc70 in inclusion bodies (Watanabe et al., 2001). Interestingly, Hsc70, but not other protein chaperones, was also found in protein inclusion bodies found in motor neurons and astrocytes from sporadic and fALS cases (Watanabe et al., 2001).

In order to perform a biochemical assessment of the proteasome acitivity and levels in tissues obtained from sporadic ALS patients as well as control cases, a series of preliminary experiments demonstrated that proteasomal proteins and their catalytic activities are quite stable post-mortem and that proteasome activities and level of 20Sα subunits in human and mouse nervous tissue are comparable under similar conditions of preservation and assay (Figure 22 and 23). Thus, assay of proteasome function and composition is realistic in human autopsy tissue at least up to 12 hours post-mortem.

All three activities of the proteasome were reduced by 55-70% in thoracic spinal cord of ALS patients relative to controls with the trypsin-like activity in ventral sections being the most affected at 46%. Interestingly, activities were reduced in dorsal spinal cord samples as well as in the ventral region where motor neurons are located. That proteasomal impairment is

present in both the ventral and dorsal regions of the spinal cord suggests that proteasomal impairment is not limited to the motor neurons and the sorrounding neighbourhood, but is a more widespread phenomenon. This could be due to the advanced state of spinal cord degeneration where other cell types (neuronal and non-neuronal) might be affected. Alternatively, proteasomal impairment as well as sporadic ALS pathogenesis might initiate simultaneously in both ventral and dorsal sections of the spinal cord.

In order to determine if the proteasome activity was generally affected in CNS tissues from ALS patients, tissue from cerebellum obtained from the same five sporadic ALS patients and four control cases was analyzed. The cerebellum shows minimal involvement morphologically in ALS (Tandan and Bradley, 1985). Also, no changes in markers of oxidative damage were detected in cerebellar regions of sporadic and fALS patients, whereas the motor cortex of these patients had increased oxidative damage (Ferrante et al., 1997). No change was detected in all three activities of the proteasome in cerebellar tissue extracts of sporadic ALS patients when compared to control cases (Figure 24A-C), evidence that the impairment of 20S/26S proteasome activity was specific to the tissue most affected by ALS.

The decrease in proteasomal activities in thoracic spinal cord of ALS patients, as seen in a decrease in the level of the β5 subunit of the 20S core and of the S5a subunit of the 19S regulatory particle of the proteasome, was determined in the ventral section of thoracic spinal cord tissue of ALS patients (Figure 25A). These proteins remained at normal levels in cerebellar tissue (Figure 25B).

Even though a focal impairment of the proteasome activity associated with a specific decrease in 20S functional subunits measured in affected spinal motor nuclei was a common finding in sporadic ALS patients and $SOD1^{G93A}$ transgenic mice, some important differences were observed. Unlike results obtained from lumbar spinal cord tissue of symptomatic $SOD1^{G93A}$ transgenic mice, there was no increase of the regulatory particle 11Sα and the

inducible subunit β5i of the immunoproteasome (Figure 25A) suggesting that either a lesser immune response is activated in tissue from sporadic ALS patients or glial reactivity and an immune response do not lead to a massive up-regulation of immunoproteasome subunits. Immunohistochemical analysis of the tissue from these patients with antibodies that label glial markers (GFAP, MAC1), immune markers (TNFα, IFNγ) and members of the immunoproteasome (11Sα, β5i) as well as other members of the ubiquitin-proteasome pathway will allow determination of the extent of the immune reaction in these tissues. A full analysis was not possible in this prelimary study because of limitations in the amount of material available to us.

Another difference between the mouse model of fALS and sporadic human ALS is that no decrease in the structural subunit of the proteasome, 20S β3, was measured in thoracic spinal cord of ALS cases, even though the expression of this subunit was decreased very early in disease pathogenesis in the lumbar spinal cord tissue of $SOD1^{G93A}$ transgenic mice. These results suggest that several converging molecular mechanisms might lead to proteasome impairment during ALS pathogenesis and a further analysis of these abnormalities is required to clarify the involvement in disease pathogenesis. Also, immunohistochemical analysis in archived paraffin-embedded slides derived from spinal cord sections from the same sporadic ALS patients and control cases were performed. Unexpectedly, several antibodies specific for α and β subunits well-characterized in murine studies (Figure 11 and 17) that immunolabel specific bands in Western blots of human spinal cord tissue extracts (Figure 25) labelled very faintly motor neurons as well as the spinal cord background (data not shown). Whether there are problems with the protocol used for immunohistochemistry and antigen retrieval, the quality of paraffin-embedded tissue or if motor neurons in human spinal cord are not immunoreactive for subunits of the 20S core remains to be determined.

Results from post-mortem tissue are very difficult to interpret since generally the spinal cord of ALS patients is affected at the morphological level and cellular differences were observed in spinal cord sections (motor neuron loss, gliosis) from these patients. Alternatively, the proteasomal levels and activities can be determined in spinal cord cerebrospinal fluid derived from early symptomatic and late symptomatic ALS patients, where there is evidence of protein misfolding, to determine the importance that the ubiquitin-proteasome pathway plays during disease progression in sporadic ALS patients.

The experiments reported in this thesis were conducted as a preliminary study to determine the relevance of proteasomal abnormalities to the more general problem of sporadic ALS and the feasibility of conducting these experiments with post-mortem tissue. The results support a full analysis of proteasome composition and assembly in a larger number of samples. The difficulty in obtaining additional control tissues makes these experiments beyond the scope of this thesis.

Figure 22

No change in proteasome activity was measured in mouse spinal cord tissue up to 12 hours post-mortem and structural subunits of the proteasome appeared intact

Following anesthetic overdose mouse spinal cord tissues were dissected and either was immediately frozen (time 0) or stored at room temperature for 3, 6 and 12 hours. Spinal cord tissue (3 mice per time point) was processed and tissue extracts were used for activity assays and SDS/PAGE gels. No changes were measured in chymotrypsin-like activity of the proteasome (A) and no difference was determined in the structural α subunits of the 20S core (B) in murine spinal cord tissue from each time point.

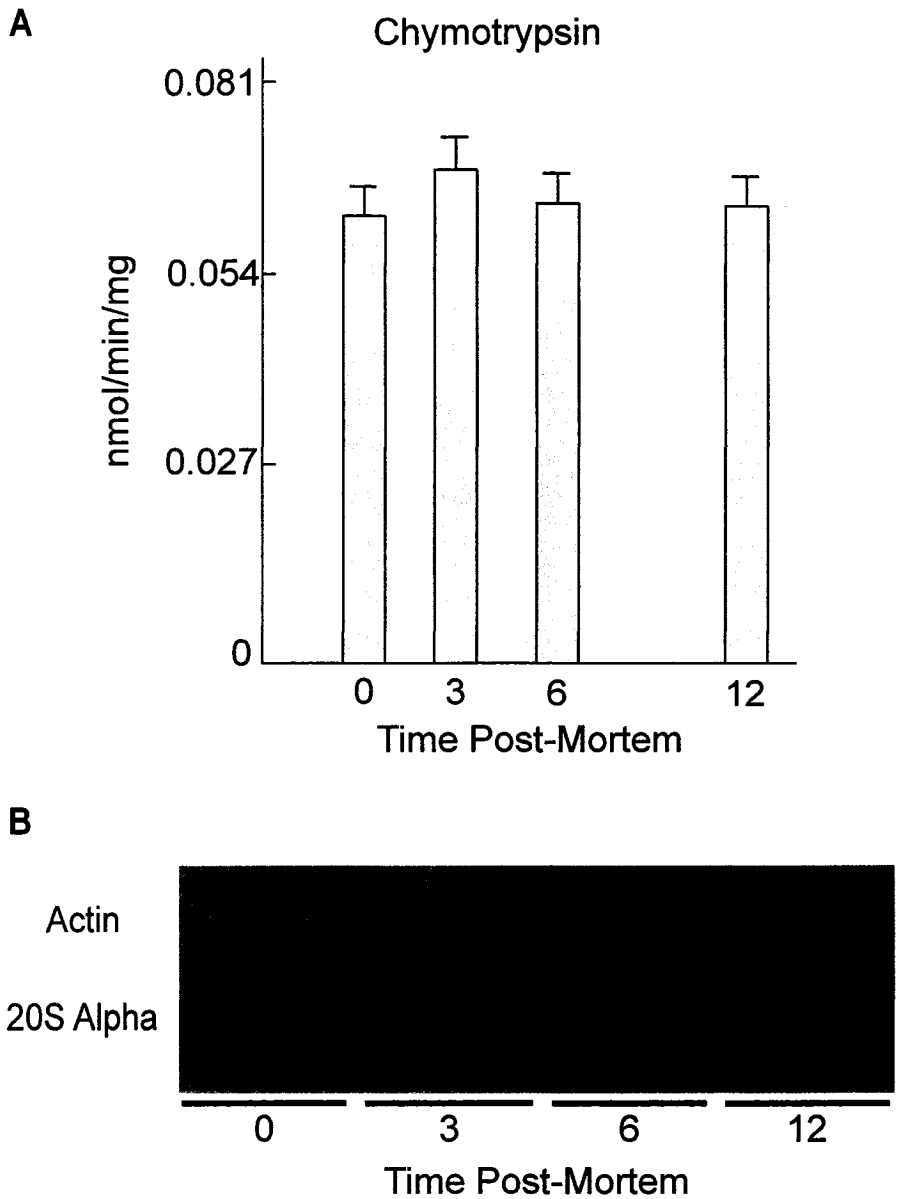

A
Chymotrypsin
0.081
0.054
0.027
0
nmol/min/mg
0
3
6
12
Time Post-Mortem
B
Actin
20S Alpha
0
3
6
12
Time Post-Mortem

Figure 22

Figure 23

Proteasomal activities measured in banked human spinal cord were similar to those of mouse spinal cord processed immediately post-mortem

Each of the three main proteasome activities was measured in frozen human thoracic spinal cord (non-ALS) obtained from Dr. Michael Strong's tissue bank of autopsied material [case was 73 yr-old male; autopsy was carried out 10 hrs post-mortem]. Samples of mouse liver (from 120 day old mice processed immediately post-mortem) were run at the same time as a technical control (see right hand panels in A,B,C). Left panels show activities of lumbar and thoracic spinal cord relative to liver in mice. Chymotrypsin-like (A), caspase-like (B), trypsin-like (C) activities of human thoracic spinal cord tissue separated in ventral and dorsal sections were quite similar to activities of lumbar and thoracic murine spinal cord. (D) Proteasomal proteins remain intact in human spinal cord tissue as detected by Western blot.

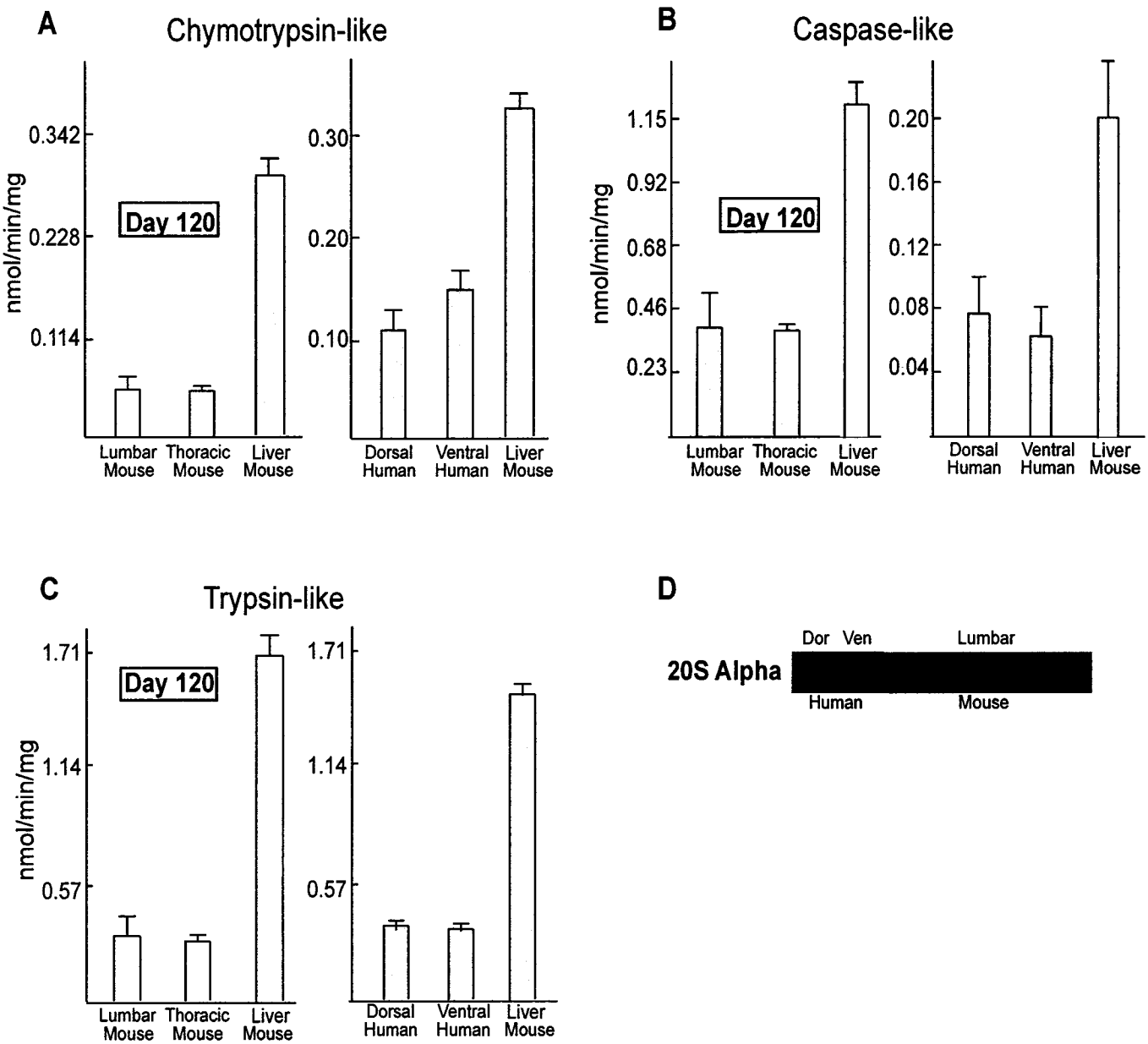

A
Chymotrypsin-like
nmol/min/mg
0.342
0.228
0.114
Day 120
Lumbar Mouse
Thoracic Mouse
Liver Mouse
0.30
0.20
0.10
Dorsal Human
Ventral Human
Liver Mouse
B
Caspase-like
1.15
0.92
0.68
0.46
0.23
0.20
0.16
0.12
0.08
0.04
C
Trypsin-like
1.71
1.14
0.57
D
Dor Ven
Lumbar
20S Alpha
Human
Mouse

Figure 23

Figure 24

Proteasome Activities are reduced in Thoracic Spinal Cord, but not Cerebellum from Sporadic ALS Patients

Chymotrypsin-like (A), caspase-like (B) and trypsin-like (C) activities expressed as nmol/min/mg after being normalized to actin levels were measured in homogenates of dorsal and ventral sections of thoracic spinal cord from five ALS patients and two control cases. Dark bars represent ventral and dorsal regions of the spinal cord of ALS patients, whereas light bars represent their age-matched, sex-matched controls. Chymotrypsin- (A), caspase- (B), and trypsin-like (C) activities were significantly reduced in both ventral and dorsal portions of the thoracic spinal cord of ALS patients. Proteasomal activities are expressed as means ± SEM (nmol/min/mg) of four independent measurements in tissue from five sporadic ALS patients and four control cases. Significantly different from LM control: (* $p<0.05$; ** $p<0.01$; *** $p<0.005$).

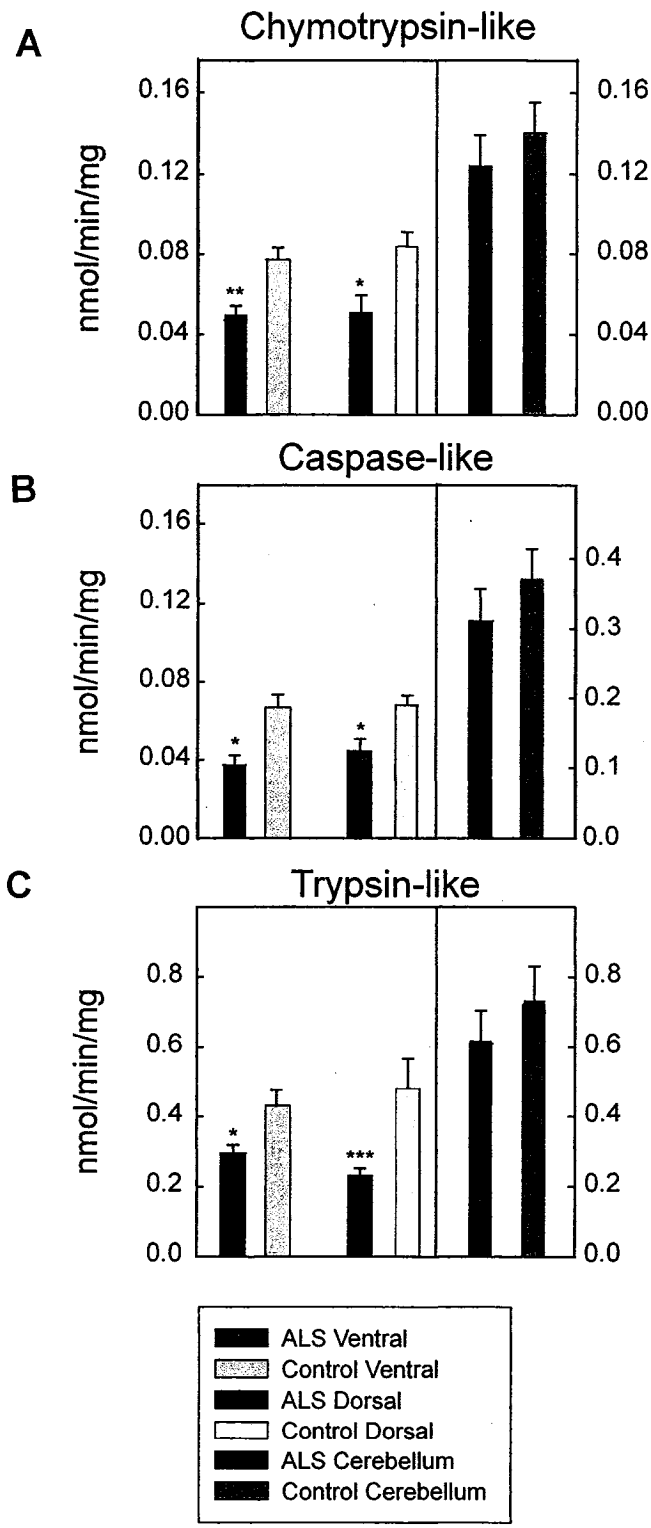

A
Chymotrypsin-like
nmol/min/mg
0.16
0.12
0.08
0.04
0.00
**
*
B
Caspase-like
0.4
0.3
0.2
0.1
0.0
C
Trypsin-like
0.8
0.6
***
ALS Ventral
Control Ventral
ALS Dorsal
Control Dorsal
ALS Cerebellum
Control Cerebellum

Figure 24

Figure 25

Levels of constitutive 20S β5 subunits and a 19S subunit were reduced in thoracic spinal cord tissue from sporadic ALS patients, but this was not associated with an increase of immunoproteasome subunits 20S β5i or 11S

Measurements of proteasome activity in post-mortem spinal cord tissue of sporadic ALS patients revealed decreased activity of the proteasome (Figure 24). SDS/PAGE and Western blotting were performed to determine levels of proteasomal subunits in spinal cord tissue and cerebellum (less affected CNS tissue) of five ALS patients and four control cases. Levels of β5 and 19S5a were decreased, reflecting significant changes in proteasome composition in an affected region of spinal cord at the end stage of ALS (panel A). However, unlike studies performed in an animal model of fALS, $SOD1^{G93A}$ transgenic mice, the levels of the inducible subunit responsible for chymotrypsin-like activity, 20S β5i, and the regulatory subunit of the immunoproteasome, 11S, were not elevated (panel A). Also, there was no change in levels of structural β3 and various α subunits of the 20S core. No changes were measured in the subunit levels of 20S core (β5, β5i, β3, α1-7), 19S (S5a) and 11Sα regulatory particles of the proteasome in cerebellar tissue of sporadic ALS patients (panel B) indicating that the changes measured in 20S β5 and 19S5a occurred only in regions affected by ALS. In panels C and D, statistical analysis of pixel density of every lane from spinal cord and cerebellar tissues obtained from five sporadic ALS patients and four control cases was normalized to actin and quantified as described in Chapter 3 (* $p<0.05$).

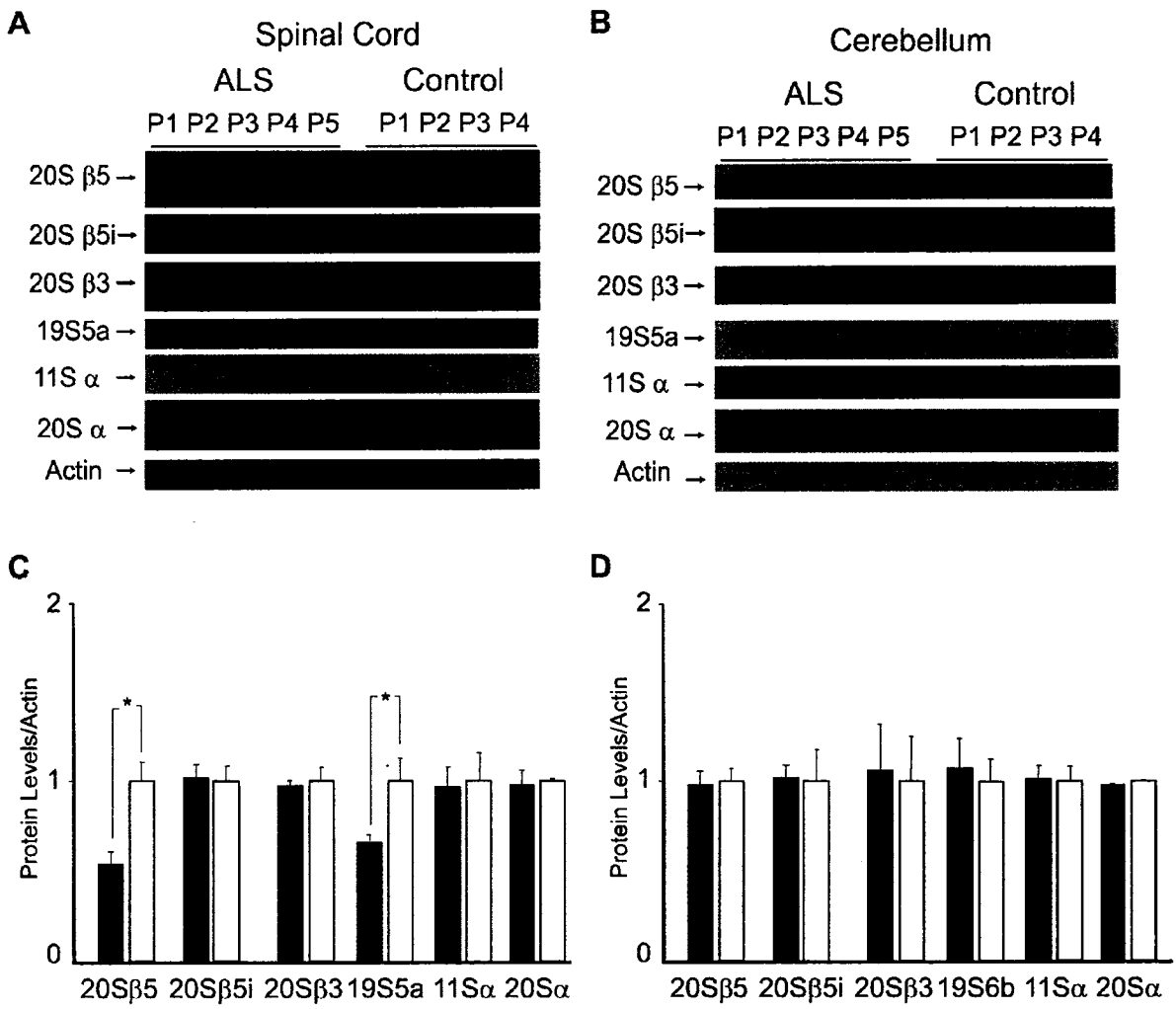

A
Spinal Cord
ALS
Control
P1 P2 P3 P4 P5
P1 P2 P3 P4
20S β5 →
20S β5i→
20S β3 →
19S5a →
11S α →
20S α →
Actin →
B
Cerebellum
ALS
Control
P1 P2 P3 P4 P5
P1 P2 P3 P4
20S β5 →
20S β5i→
20S β3 →
19S5a →
11S α →
20S α →
Actin →
C
Protein Levels/Actin
2
1
0
*
*
20Sβ5 20Sβ5i 20Sβ3 19S5a 11Sα 20Sα
D
Protein Levels/Actin
2
1
0
20Sβ5 20Sβ5i 20Sβ3 19S6b 11Sα 20Sα

Figure 25

Figure 26

Increased levels of Hsp70 suggests a dysfunction of the ubiquitin-proteasome pathway

A. Levels of Hsp70, but not Hsp40 were found to be increased in spinal cord, but not in cerebellar tissue of sporadic ALS patients. As in murine studies, proteasome impairment coincides with increased expression of the protein chaperone, Hsp70. Quantitations of bands from representative Western blots in (A) were performed as described in Chapter 3 and averages of these bands ± SEM are presented in panel B (* $p<0.05$).

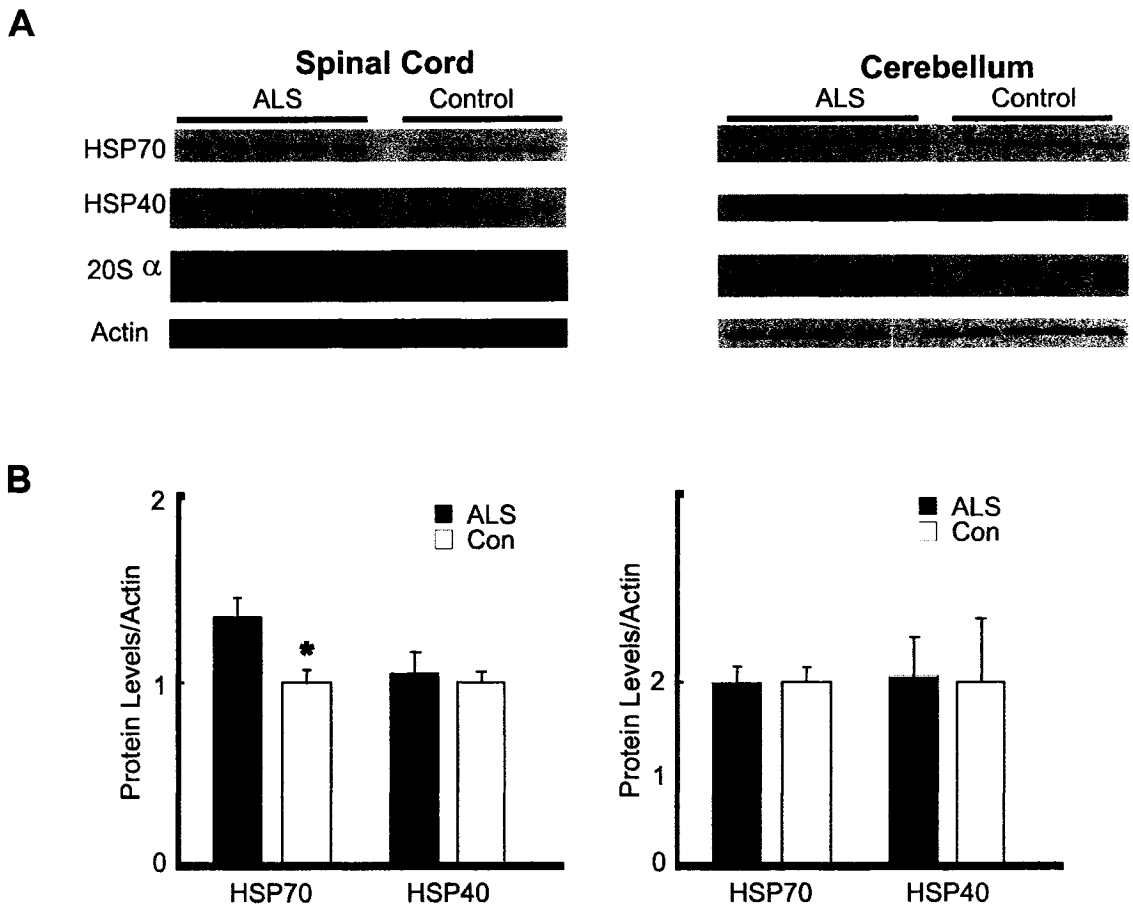

A
Spinal Cord
ALS
Control
HSP70
HSP40
20S α
Actin
Cerebellum
ALS
Control
B
Protein Levels/Actin
2
1
0
ALS
Con
*
HSP70
HSP40
Protein Levels/Actin
2
1
0
ALS
Con
HSP70
HSP40

Figure 26

Chapter 7 General Discussion

In this chapter, the evidence that proteasomal impairment contributes to the pathogenesis of fALS will be summarized and discussed in light of recent advances illustrating the essential role that the ubiquitin-proteasome pathway plays in the pathogenesis of several neurodegenerative disorders. Similarities and differences in approach, methodology, and results obtained in these studies in comparison to those described in this thesis will be presented. The consequences and potential mechanisms of proteasome impairment will be discussed, including the impact that focal dysfunction of the proteasome may play in mediating the toxic-gain-of function in a specific cellular and tissue setting. Finally, the efficacy of treatments envisaged to relieve the impairment of the ubiquitin-proteasome pathway in treating ALS as well as other neurodegenerative disorders will be considered.

7.1 Proteasome Impairment Contributes to fALS Pathogenesis

The following paragraphs summarize the evidence supporting the involvement of the ubiquitin-proteasome pathway in fALS:

7.1.1 The ubiquitin-proteasome pathway is important for degrading mutant SOD1

Treatment of cell lines overexpressing mutant SOD1 with proteasomal inhibitors (MG132 and lactacystin), but not calpain inhibitors, led to increased levels of mutant, but not $SOD1^{WT}$ (Hoffman et al., 1996; Johnston et al., 2000). The E3 ligase, dorfin, identified in ubiquitinated inclusion bodies of sporadic and fALS patients, and CHIP ubiquitinate mutant SOD1, but not WT SOD1, and promotes its rapid degradation by the 26S proteasome (Niwa et al., 2002) (Urushitani et al., 2004). Overexpression of dorfin rescued neuronal cell lines from mutant SOD1 toxicity (Niwa et al., 2002). Overexpression of these proteins in animal

models of fALS is crucial to determine if these E3 ligases have an impact in this neurodegenerative disease progression. However, a recent study has shown that recombinant SOD1 mutants do not need to be ubiquitinated. They are good substrates for the 20S proteasome *in vitro,* particularly as metal deficient monomers lacking the intrasubunit disulfide bond (Di Noto et al., 2005). Also, preferential degradation by the 20S proteasome has been previously demonstrated when $SOD1^{WT}$ recombinant protein was oxidatively damaged (Salo et al., 1990).

7.1.2 Proteasome activities are reduced in some cell lines and in lumbar spinal cord of transgenic mice overexpressing mutant SOD1, but not $SOD1^{WT}$

Stable and transient overexpression of mutant and $SOD1^{WT}$ in various cell lines led to decreased (Urushitani et al., 2002; Hyun et al., 2002b; Allen et al., 2003), increased (Casciati et al., 2002; Aquilano et al., 2003) or unchanged (Lee et al., 2001) total proteasomal activities. A decrease of the proteasome specific activities as normalized to structural α subunits of the 20S core was measured in NIH3T3 cell lines (Figure 10) (Kabashi et al., 2004). A number of factors could influence the disparate results depending on cell lines and conditions including differences in conditions promoting misfolding of mutant SOD1 and responsiveness of ubiquitin-proteasome components and other protective pathways.

As reported in this thesis, abnormalities in proteasome activities and structure were identified in vulnerable tissue of mutant SOD1 transgenic mice at three stages of disease pathogenesis defined by the extent of glial activation (Figure 4): P45 with minimal glial activation; P75 when the glial response has initiated but no signs of behavioural symptoms and motor neuronal death are present (Chiu et al., 1995), and at symptomatic stage where there is massive gliosis and over 50% of motor neurons have been lost. Even when levels of structural α subunits of the 20S core remained unchanged, a specific decrease of the

chymotrypsin-like activity of the proteasome was measured within the lumbar spinal cord (Figure 8A), but not in the thoracic region of the spinal cord, a region less affected during the course of ALS pathogenesis (Chiu et al., 1995), and/or in whole spinal cord extracts of $SOD1^{G93A}$ transgenic mice (Kabashi et al., 2004). By P75, all three proteolytic activities of the proteasome were severely reduced at about 50% of the proteasomal activities in $SOD1^{WT}$ transgenic mice and their age-matched non-transgenic littermates (Figure 8A,C,D).

7.1.3 Impaired proteasome activity occurs in tissue most vulnerable to disease

At this stage of the disease no changes were measured in the total and/or specific activities of the 20S/26S proteasome in the thoracic region of the spinal cord (Figure 7A,C,D and 8A,C,D) and in whole spinal cord tissue (Kabashi et al., 2004; Cheroni et al., 2005; Puttaparthi and Elliott, 2005).

7.1.4 Proteasome impairment corresponds to the presence of SDS-insoluble, high-molecular weight mutant SOD1 species

These species were ubiquitinated and were enriched in lumbar spinal cord (Figure 6). Also, levels of the protein chaperone, Hsp70, which is up-regulated upon proteasome inhibition in spinal cord cultures (Batulan et al., 2003) and rat substantia nigra (Ahn and Jeon, 2006) were found to be specifically increased in lumbar spinal cord (Figure 12).

7.1.5 Proteasome levels decrease with disease progression in lumbar spinal cord of $SOD1^{G93A}$ mice

Finally, even though motor neurons express high levels of 20S α subunits compared to other cell types in the spinal cord, the proteasome levels progressively decreased in surviving motor neurons within the lumbar spinal cord of $SOD\text{-}1^{G93A}$ mice relative to the surrounding

neuropil (Figure 11 and 17).

These results demonstrate that there is a focal dysfunction of proteasome activity early in disease pathogenesis in lumbar spinal cord and later as expression of 20S subunits is depleted in motor neurons of a fALS mouse model (Kabashi et al., 2004). However, the cell types involved and the functional consequences remain to be elucidated. Bendotti's group is attempting to answer this question by crossing $SOD1^{G93A}$ mice with Ub^{G76V}-GFP transgenic mice. As discussed in Section 1.2.6.5, a limitation of the GFP reporters of proteasomal function is that significant loss of activity may be required before GFP accumulates to readily visible levels ((Dantuma et al., 2000; Bennett et al., 2005) and how long various spinal cord cells can live with this level of functional impairment is not known.

7.1.6 Antibody-based techniques identify structural defects in proteasome composition

Decreased levels of two 20S β subunits, β5 catalytic subunit and the structural 20S β3 subunit, of the proteasome occurred early in lumbar but not in thoracic spinal cord of $SOD1^{G93A}$ mice and coincided with impairment of proteasome activity (P45 and 75) (Figure 13 and 14).

7.1.7 Proteasome activities are reduced in spinal motor nuclei of sporadic ALS patients

As presented in Figure 24 proteasome impairment measured as a significant decrease of all three major activities of the proteasome occurred in both ventral and dorsal sections of thoracic spinal cord (Figure 24A,B,C right panels) but not in cerebellar tissue (Figure 24A,B,C left panels).

7.1.8 Decreased subunit expression coincides with proteasome impairment in sporadic ALS patients

As presented in Figure 25, impairment of the activity coincided with a specific decrease in protein levels of 20S β5 and 19S5a subunits in spinal cord tissue. No changes in other subunits of the proteasome were measured in spinal cord tissue and cerebellum. Also, an increase in Hsp70 levels in spinal cord suggests an impairment of the ubiquitin-proteasome pathway (Figure 26).

7.1.9 Proteasome impairment is not directly caused by mutant SOD1 expression

In cell lines, fluorescent vital imaging techniques showed that mutant SOD1 aggregates form inclusion bodies that stably associate with subunits of the proteasome causing progressive impairment of its activity (Matsumoto et al., 2005) as determined using a GFP-Ub reporter (Bennett et al., 2005). Using 2-D blue native/SDS-PAGE to assess the composition of proteasomes in lumbar spinal cord tissue of $SOD1^{G93A}$ mice, mutant SOD1 was not found to be directly associated with the proteasome complexes (Figure 21A), and immunodepletion of mutant SOD1 from samples did not increase proteasome activities in homogenates of these tissues. Although these experiments are not conclusive, they do not support the hypothesis of "choking" of the proteasome by mutant protein as was suggested by studies in cell lines (Bennett et al., 2005). Rather our results point to structural alterations associated with decreased proteasome activity.

7.1.10 No evidence of transcriptional impairment of proteasomal subunits and malformation of proteasomal complexes in affected murine tissue

None of the changes in levels of proteasomal proteins resulted from decreased gene transcription, as mRNA levels were at control levels (Figure 18 and 19). In accordance with our results, recently, Kato and colleagues demonstrated that in the wobbler mouse (model of motor neuron disease – see Section 1.1.5.2) but not in mutant SOD1 transgenic mice, mRNA

level of the β7 subunit of the 20S core was decreased in spinal cord tissue of pre-symptomatic mice (Perrin et al., 2005). Nor were there increases in levels of unassembled β subunits in 20S and 26S proteasome complexes measured in lumbar spinal cord of SOD-1^{G93A} transgenic mice (Figure 20A). Purification of the proteasome and subunit analysis might detect post-translational modifications that lead to proteasome impairment in affected tissue of mutant SOD1 transgenic mice.

7.1.11 Not a simple matter of immune response

Early in disease pathogenesis, the decrease in β5 was not compensated for by an increase in the inducible immunoproteasome subunit, β5i. However, at symptomatic stage, levels of the inducible subunit β5i and the regulatory particle 11Sα of the immunoproteasome were increased (Figure 15), due to the inflammatory response at this stage of the disease as previously demonstrated in the spinal cord of SOD-1^{G93A} mice (Cheroni et al., 2005; Puttaparthi and Elliott, 2005).

7.1.12 Are motor neurons particularly sensitive to proteasome inhibition?

In primary spinal cord cultures and organotypic spinal cord slices, treatment with the proteasome inhibitor, lactacystin, led to selective motor neuronal death (Urushitani et al., 2002; Tsuji et al., 2005). These studies have come under controversy, since long-term low-level proteasomal inhibition by lactacystin led to degeneration of both interneurons and motor neurons at the same rate (Vlug and Jaarsma, 2004). Also, proteasome inhibition by lactacystin in organotypic slices and fibroblasts derived from $SOD1^{G93A}$ transgenic mice caused increases in SOD1-positive inclusion bodies and SDS-insoluble mutant SOD1 (Puttaparthi et al., 2003; Turner et al., 2004). These data suggest that mutant SOD1 toxicity can be amplified by inhibiting the proteasome. However, it is important to determine whether treatment of spinal

cord tissue of rodents with proteasome inhibitors would lead to selective death of motor neurons and a neurodegenerative disorder similar to the course of the disease in mutant SOD1 transgenic mice.

7.1.13 Proteasome activity in models of fALS and tissue from sporadic ALS patients: Specific mechanisms of impairment?

As previously discussed in Chapter 6, impairment of the proteasome activity was measured in affected tissue of sporadic ALS patients as well as in an animal model of fALS. However, certain differences are worth discussing. No increases in levels of immunoproteasome components were observed in sporadic ALS patients, even though elevation of immunoproteasomes at late stages of the disease is a well-documented feature of the $SOD1^{G93A}$ transgenic mouse (Kabashi et al., 2004; Cheroni et al., 2005; Puttaparthi and Elliott, 2005). Whereas levels of the subunit harbouring the chymotrypsin-like activity of the proteasome, 20S β5, were decreased both in tissues from sporadic ALS patients and in an animal model of fALS, levels of the structural β3 subunit were unaffected in sporadic ALS patients but were altered very early in disease pathogenesis in $SOD1^{G93A}$ transgenic mice. Finally, a recent report suggested that expression of the 20S core is increased in degenerating motor neurons from ALS patients (Mendonca et al., 2006). These data are contrary to studies in murine models of fALS that illustrated a severe depletion of proteasomal subunits in lumbar motor neurons of symptomatic $SOD1^{G93A}$ transgenic mice.

As it will be discussed in the next section, it is possible that several molecular mechanisms of disease can lead to proteasome impairment. A better understanding of proteasome biogenesis and the development and maintenance of proteasome activity in mammalian cells and tissues will certainly aid in unravelling these mechanisms of proteasome impairment in ALS and other neurodegenerative disorders and provide avenues of

pharmacological treatments designed to reverse proteasome impairment in ALS pathogenesis.

7.2 Role of the Ubiquitin-Proteasome Pathway in Neurodegenerative Disorders and Aging

The first direct evidence for involvement of the ubiquitin-proteasome pathway in neurodegenerative disorders was identification of parkin, an E3 ligase whose mutations cause autosomally recessive PD (Kitada et al., 1998). Other genes that participate in this pathway and are mutated in human disorders include two E3 ligases, E6-AP and VHL, which can cause respectively Angelman syndrome and the von-Hippel-Lindau disease (Kishino et al., 1997; Kamura et al., 1999). Also, UCHL-1, a gene which is mutated in a rare form of dominant PD (Lincoln et al., 1999), encodes a protein that was shown to function simultaneously as a DUB enzyme and an E3 ligase (Liu et al., 2002). Despite the numerous studies, the protein substrates that these E3 ligases are involved in degrading are not well defined. Recently, gigaxonin, the product of a gene mutated in Giant Axonal Neuropathy, was found to bind to an E1 at its N-terminus and to MAP1B through its C-terminal domain gigaxonin is involved in the degradation of MAP1B (Wang et al., 2005c), which is impaired when gigaxonin is mutated or ablated (Allen et al., 2005). Currently, no mutations in genes that would encode members of the ubiquitin-proteaome pathway have been identified in fALS or "sporadic" ALS patients.

There is a large body of evidence to suggest that the proteolytic capacity of the proteasome is impaired in aged organisms. Decreases in proteasome activity have been reported in various mammalian tissues (Chondrogianni and Gonos, 2005), such as spinal cord (Keller et al., 2000a), brain regions (Abd El Mohsen et al., 2005), retina (Louie et al., 2002), heart (Bulteau et al., 2002), liver (Shibatani et al., 1996), and proteasome impairment has been shown in senescent human fibroblasts (Sitte et al., 2000b). However, little is known

about the molecular mechanisms that lead to this proteasome impairment. Progressive overload of the ubiquitin-proteasome pathway due to increased errors in translation and transcription and increased oxidative modifications of proteins has been proposed (Grune et al., 2004). Severely oxidized proteins, including glycated proteins or proteins conjugated by HNE, can inhibit the 20S core of the proteasome (Carrard et al., 2003). Also, fluorescent pigments of oxidatively damaged aggregated polymers, such as lipofuscin and ceroid have been shown to inhibit all three activities of the proteasome (Sitte et al., 2000a). It has been proposed that these cross-linked proteins cannot be properly unfolded and block the entrance gate of the proteasome, although no *in vivo* data exist to support this hypothesis (Kisselev et al., 2002). Even though there is evidence that glycated (Kato et al., 2000), HNE-modified (Perluigi et al., 2005), lipofuscin and ceroid (McHolm et al., 1984) accumulate in ALS with disease progression in affected tissue it remains to be determined whether their accumulation leads to proteasome inhibition and impaired degradation of other important substrates, and whether this occurs early in ALS disease pathogenesis.

Oxidative damage to 20S subunits has also been proposed as a possible mechanism for proteasome impairment during aging (Chondrogianni et al., 2003). Indeed, HNE-modifications of several α and β subunits of the 20S core that can impair proteasome activity have been demonstrated in aged tissue and certain subunits were shown to be more sensitive to these modifications (Bulteau et al., 2000). Indeed, Keller and colleagues demonstrated HNE-modification of β, but not α subunits occurs in aged spinal cord of rats (Keller et al., 2000a). Finally, decreased expression of three functional subunits β1, β2, β5, but not other subunits of the 20S and 19S components in human fibroblasts occurs with aging and correlates with impairment of proteasome activity (Chondrogianni et al., 2003). The authors suggested that these subunits are the rate-limiting ones for proteasome activity, and recently they reported that stable overexpression of β5 can lead to increases in mRNA of other

functional β subunits, their assembly into proteasomal complexes and increased rates of proteasome activities (Chondrogianni et al., 2005). It remains to be determined if the functional β subunits are the rate-limiting subunits in CNS tissue and if overexpression of these subunits can relieve proteasome impairment in fALS affected cells and tissues and lower the toxicity of mutant SOD1 and possibly delay the onset of symptoms in mutant SOD1 transgenic mice. As previously discussed, a specific decrease in 20S proteasome levels of β subunits in lumbar spinal cord of mutant SOD1 transgenic mice contributed to proteasome impairment. However, our characterization of 20S and 26S complexes in the lumbar spinal cord demonstrated that both α and β subunits are present as monomers (Figure 20), therefore no evidence of β subunits being rate-limiting was found in murine spinal cord tissue extracts.

Kirino and his group (Asai et al., 2002) were the first to report a proteasomal dysfunction in a murine model of brain stroke. Occlusion/reperfusion leads to delayed neuronal death in the CA1 area of the hippocampus. Proteasome activity was decreased by 60% in a general area of the CNS (frontal cortex and hippocampus) right after occlusion, but the activity was restored during the reperfusion period. However, in the CA1 area the activity of the proteasome remained reduced leading these researchers to hypothesize that proteasome inhibition in the affected region causes neurodegeneration (Asai et al., 2002). We demonstrated that in a murine model of ALS, proteasome impairment occurs early in disease pathogenesis and is restricted to the lumbar spinal cord, the region most affected by the disease (Kabashi et al., 2004).

Further studies in animal models of neurodegenerative disorders confirmed the important role that the ubiquitin-proteasome pathway plays in neurodegenerative disorders. Proteasome impairment and a corresponding increase in autophagic pathways were determined in the sciatic nerve from mice that have a duplication of peripheral myelin protein 22 (PMP22), a mouse model of hereditary neuropathy (Fortun et al., 2005; Fortun et al.,

2006). On the other hand, proteasome activity was reported not to be affected in certain animal models of neurodegenerative disorders. No changes in proteasome activity, levels, composition and assembly were observed prior to disease onset (gliosis) in diseased tissue from mice that overexpressed mutant α-synuclein (Martin-Clemente et al., 2004), whereas another report showed significant impairment of proteasome activity in affected tissue (substantia nigra) of symptomatic mice overexpressing mutant α-synuclein (Chen et al., 2006). Also, in two murine models of polyglutamine disorders, R6/2, which expresses a fragment of mutant huntingtin, and mutant SCA7, no increase of proteasome activity was measured early in the disease (Diaz-Hernandez et al., 2003; Bowman et al., 2005). At symptomatic stage, an increase in activity of the proteasome as well as levels of its inducible subunits was possibly due to the high rate of gliosis and immune response present at this stage in affected tissue of CNS (Valera et al., 2005). These results demonstrate that the ubiquitin-proteasome pathway might be less affected in several neurodegenerative disorders. Interestingly, a mouse model expressing full-length mutant huntingtin exhibits impaired proteasomal activity in affected areas (Seo et al., 2004) suggesting that in different models of neurodegenerative disorders the proteasome activity might be affected at various rates or through different mechanisms. More studies in mouse models of neurodegenerative disorders and better techniques to directly measure *in vivo* proteasome activity are needed to determine if the ubiquitin-proteasome pathway is directly affected by mutant, post-translationally modified and/or misfolded proteins.

Proteasome impairment has recently been shown to directly cause neurodegeneration. Systemic treatment of mouse and rat brain with three selective proteasome inhibitors, lactacystin, epoximicin and Z-Ile-Glu(O*t*Bu)-Ala-Leu-al (PSI) led to selective degeneration of substantia nigral and striatal dopaminergic neurons and development of PD-like features in these mice. This represents additional strong piece of evidence supporting this hypothesis

(McNaught et al., 2002; McNaught et al., 2004). These findings have come under strong debate recently since there are several reports that support (McNaught and Olanow, 2006; Zeng et al., 2006a) and argue against (Manning-Bog et al., 2006; Bove et al., 2006) nigrostriatal death upon proteasomal inhibition. Also, continuous injections of MPTP lead to a PD-like model in rodents, inhibited the proteasome activity (Fornai et al., 2005). With respect to ALS, it will be important to determine whether these proteasome inhibitors would cause degeneration of motor neurons, cells that have been shown in cultures to be particularly vulnerable to proteasome inhibition.

As introduced in Chapter 6, similar to the situation in ALS patients, immunohistochemical analysis has shown that various members of the ubiquitin-proteasome pathway are sequestered in inclusion bodies of AD, PD and HD with high frequency (Ciechanover and Brundin, 2003). Recently, a ubiquitin variant, ubiquitin-B+1 (UBB+1) has been demonstrated to accumulate in neurons of patients with Alzheimer's disease (AD) and other pathologies associated with tau-positive inclusion bodies but not in pathologies with α-synuclein-positive inclusion bodies (De Vrij et al., 2001). This ubiquitin variant was recently shown to directly inhibit 26S proteasome activity and induce HSP expression in cell lines (Hope et al., 2003). Since there is evidence of tau deposition, it would be interesting to determine if this ubiquitin variant is accumulated in tissue from ALS patients (Strong et al., 2006).

7.3 Consequences of Proteasomal Dysfunction in ALS Pathogenesis

As presented in the First Chapter of this thesis, several hypotheses attempt to explain the toxic gain of function that mutant SOD1 might exert to cause fALS. In this section, the role that a dysfunctional ubiquitin-proteasome pathway may play in these molecular

mechanisms will be briefly discussed suggesting the central role that proteasomal-dependent degradation plays in the pathogenesis of ALS and other neurodegenerative disorders.

Studies by Davies and colleagues have shown that most oxidatively modified protein substrates (including SOD1) are degraded by the proteasome (Grune et al., 2004). Thus, the proteasome plays a crucial role in regulating oxidative damage, with preservation of proteasome function preventing oxidative stress, and proteasome inhibition being a mediator of oxidative stress. Mild, physiological oxidative stress leads to unfolding of proteins and exposure of hydrophobic patches that can be selectively recognized by the 20S proteasome and degradation of these substrates could occur without a need for ubiquitin targeting or ATP activation. However, severely oxidized, aggregated, and crosslinked proteins are poor substrates for degradation and eventually inhibit the proteasome (Grune et al., 2005). For this reason generation of reactive oxygen species might corresponds to a decline in proteasome activity in aged tissue and could play an important role in the pathogenesis of neurodegenerative disorders, resulting in the progressive accumulation of oxidatively damaged protein aggregates that eventually contribute to cellular dysfunction and senescence.

The high level of glutamatergic input to motor neurons is a contributing factor to their vulnerability to toxicity, in part due to the presence of calcium-permeable AMPA receptors (Carriedo et al., 1996; Roy et al., 1998). Formation of inclusions containing mutant SOD1 and death of cultured motor neurons are reduced by AMPA receptor blockers (Roy et al., 1998). Little is known about how proteasome dysfunction in neurons can contribute to glutamatergic toxicity. However, proteasome function is required for proper synaptic plasticity, including internalization of GluR2 receptors (Ehlers, 2003). Recently, PSD95, a major scaffolding protein that tethers NMDA- and AMPA-type glutamate receptors, was found to be a proteasomal substrate (Colledge et al., 2003). NMDA receptor activation leads to rapid ubiquitination and degradation of PSD95 and selective internalization of GluR2 receptors

(Colledge et al., 2003). As presented in section 1.2.2.1, levels of GluR2 contribute to selective vulnerability of motor neurons and might play an important role in ALS pathogenesis. The proteasome could play an important role in this molecular pathway since an impairment of proteasome function early in disease pathogenesis would lead to improper internalization of GluR2 receptors. Improper GluR2 recycling and internalization due to proteasome malfunction in ALS pathogenesis requires more attention. Also, other molecular pathways that would link proteasome degradation and glutamate toxicity in motor neurons need to be studied.

Motor neurons in $SOD1^{G93A}$ and $SOD1^{G37R}$ transgenic mice exhibit mitochondrial abnormalities (Chiu et al., 1995; Kong and Xu, 1998; Bendotti et al., 2001; Jaarsma et al., 2001; Jung et al., 2002; Higgins et al., 2003; Liu et al., 2004), although these changes are not prominent in mice expressing lower copies of these mutant or another mutant $SOD1^{G85R}$(Bruijn et al., 1997). Impaired mitochondrial function and vacuolation have been attributed to accumulation of mutant SOD1 in the intermembrane space (Jaarsma et al., 2000; Higgins et al., 2003; Liu et al., 2004), but the involvement of proteasomal dysfunction deserves investigation. Low level, non-toxic proteasome inhibition in cell lines can disrupt mitochondrial homeostasis through inhibition of mitochondrial complex I and complex II activities and increased generation of reactive oxygen species (Ling et al., 2003; Sullivan et al., 2004). Injection of two pharmacological agents, MPTP and rotenone, known to disrupt mitochondrial homeostasis in primates and rodents leads to selective degeneration of substantia nigra neurons and Parkinsonian-like syndromes (Langston et al., 1983; Betarbet et al., 2000). Both MPTP and rotenone were found to inhibit proteasome activity in affected tissues. Interestingly, treatment with rotenone leads to reduction of functional 20S β subunits (Wang et al., 2006a), whereas MPTP is associated with decreases in protein expression of 20S α subunits, but not β subunits and regulatory particles (19S and 11S) (Zeng et al., 2006b)

suggesting that several structural changes of the proteasome complex can lead to impairment of its activity.

Impaired proteasome functioning would lead to activation of other proteolytic pathways, including calpains, caspases and autophagy. Since cancer cells are more susceptible to apoptosis, treatment with proteasome inhibitors leads to up-regulation of caspases and selective apoptosis (Adams, 2003). For this reason, bortezomib, a proteasome inhibitor has proved beneficial in animal models and is already in clinical trials for cancer patients (Princiotta et al., 2001; Cavo, 2006). Subunits that participate in formation of autophagosomes, a less selective protein and organelle degradatory machinery, are also up-regulated upon proteasome impairment (Ding et al., 2003) and autophagy might play a role in the dissolution of ubiquitinated inclusion bodies in neurodegenerative disorders (Rideout et al., 2004). Recently, two groups demonstrated that inhibition of basal autophagy, by eliminating genes in the CNS responsible for formation of autophagosomes, led to neurodegeneration in mice (Hara et al., 2006) (Komatsu et al., 2006). This evidence suggests that autophagy might play a role in ALS pathogenesis, particularly if proteasomal pathways for proteolysis are not operating optimally. This needs to be investigated.

Proteasome malfunction that results in accumulation of misfolded proteins can trigger ER stress (VanSlyke and Musil, 2002). The ER stress response may play an important role since this organelle, apart from processing secretory and plasma membrane targeted proteins, also regulates Ca^{2+} storage and signalling (Turner and Atkin, 2006). There is mounting evidence that ER stress is up-regulated pre-symptomatically in animal models of fALS. An ER chaperone, Grp78 was found to be up-regulated and associated *in vivo* with neuronal protein deposits in $SOD1^{G93A}$ transgenic mice (Tobisawa et al., 2003). Also, proteomic studies found the ER-resident protein disulfide isomerase (PDI) family members to be up-regulated in lumbar spinal cord of $SOD1^{G93A}$ transgenic rats (Atkin et al., 2006). Fragmentation of the

Golgi apparatus is a major pathological feature of ALS and has been reported in motor neurons of sporadic and fALS as well as animal models of fALS (Fujita et al., 2000; Stieber et al., 2000). It is not well understood which forms of mutant SOD1 (soluble, insoluble, ubiquitinated), if any, lead to Golgi fragmentation in motor neurons, but in cell lines, mutant SOD1 has been demonstrated to impair retrograde axonal transport, which results in dispersal of the Golgi (Atkin et al., 2006). Interestingly, it was demonstrated recently that mutant SOD1 binds to components of neurosecretory vesicles, chromogranins, and is exported outside the neurons where it can trigger activation of microglia (Urushitani et al., 2006). However, the role of chromogranin-mediated secretion *in vivo*, at what point in ALS pathogenesis it occurs and whether the ubiquitin-proteasome pathway plays a role in this response remains to be determined.

As introduced in Section 1.2.4, disruption of axonal organization and transport is an important aspect of ALS pathogenesis. The ubiquitin-proteasome pathway and its degradatory capacity play very important roles in the process of axonal degeneration as demonstrated in Wld^S, an animal model in which Wallerian axonal degeneration following distal nerve trans-section occurs more slowly than normal (Raff et al., 2002). Experiments in this strain of mice have demonstrated that axonal degeneration is not a passive process, but involves several degradatory pathways, including caspases, 26S proteasomes and autophagy (Korhonen and Lindholm, 2004). Studies in mutant SOD1 mice, including a recent report by Caroni and colleagues, demonstrated that in $SOD1^{G93A}$ transgenic mice motor neuronal axonal abnormalities occur prior to motor neuronal death early during disease pathogenesis. Most large axons of motor neurons in the lumbar spinal cord are lost and become abruptly denervated between post-natal day 48 to 55 (Pun et al., 2006). Since the activity of the proteasome as measured in results reported in this thesis is first decreased at day 45 and is

severely impaired by day 75, it is important to determine the role that the ubiquitin-proteasome pathway might play in axonal degeneration during ALS pathogenesis.

7.4 Future Perspectives in the Role of the Ubiquitin Proteasome Pathway in ALS

In this section of the General Discussion, several strategies to unravel the gain of function through which mutant SOD1 causes toxicity in motor neurons that eventually leads to their demise and whether the proteolytic capacity of the proteasome plays a central role in this gain of function will be discussed. Understanding these mechanisms of toxicity will be necessary to design proper pharmaceutical treatments for sporadic and familial ALS patients.

Studies using recombinant mutant SOD1 have demonstrated that this protein is unstable, making it prone to aggregation and post-translational modifications, and can become insoluble (Hart, 2006). There is an immediate need to carry out large-scale proteomics studies in animal models of fALS as well as in affected tissue of sporadic ALS patients. SDS-insoluble, high-molecular weight species of mutant SOD1 are detected in spinal cord of $SOD1^{G93A}$ transgenic mice very early in disease pathogenesis (post-natal day 30) (Johnston et al., 2000). Therefore, the biophysical characterization of mutant SOD1 should be carried out at various stages of disease progression in affected tissues of animal models in order to dissociate pathogenic from pathologic mutant SOD1. These experiments are currently underway in our laboratory as well as in others (Agar, Kabashi et al. manuscript in preparation). Also, techniques to purify and characterize by mass spectrometry various forms of mutant SOD1 that have been associated with toxicity are required. These forms include non-ionic detergent insoluble SOD1, high molecular weight SDS-insoluble species species, components of protein aggregates trapped in filter paper, proteins immunopurified with antibodies that recognize oxidative damage markers (HNE), and proteolytic fragments of mutant SOD1 that have been demonstrated to be present in spinal cord tissue of mutant SOD1

transgenic mice. Finally, characterization of SOD1-positive inclusion bodies that develop 2-3 days following microinjection of mutant SOD1 genes in primary cultured motor neurons would be very informative to determine what other proteins are trapped in these inclusions and whether these polymers undergo any post-tranlational modifications.

Once isolated and identified, it could be determined whether various forms of mutant SOD1 can impair the activity of the protein quality control systems, protein chaperones and the ubiquitin-proteasome pathway. Kinetic analysis of the rate of proteasome impairment could be carried out by adding different forms of monomers and polymers to commercially available or purified 20S proteasomes, as previously described with aggregated α-synuclein, which directly inhibits 20S but not 26S proteasome activity *in vitro* (Snyder et al., 2003). Also, the mutant SOD1 variants can be added to spinal cord extracts and the effect on proteasomal and chaperoning activity determined.

There are technical limitations to these studies since the amount of material necessary to carry out these experiments is quite large and the amount of mutant SOD1 that can be immunopurified from affected tissue early in disease pathogenesis is quite small. Necessary steps to improve protein analysis by mass spectrometry as well as purification methods that would yield more material are required to carry out most of the studies proposed above.

Experiments should also be carried out in tissue from sporadic ALS patients to identify post-translationally modified, misfolded proteins that could contribute to disease. Antibodies specific to HNE-modified proteins, glycated proteins and other forms of oxidative damage can be used to determine which proteins are post-translationally modified. Furthermore, the time point in disease pathogenesis at which modification of these protein substrates occurs can be assessed in affected tissue of animal models of fALS.

Another question that could be addressed by proteomic studies is whether subunits of the ubiquitin-proteasome pathway are post-translationally modified in affected tissue early in

disease pathogenesis. Since an impairment of the proteasome associated with a decrease of β5 subunit of the 20S core occurs as early as post-natal day 45, these modifications can be prominent very early in disease pathogenesis. Previous studies that have demonstrated post-translational modifications of proteasome subunits involved semi-purification of proteasome complexes by gradient centrifugation, electrophoretical separation of the subunits using 2D gels and finally, analysis of the resolved subunits by mass spectrometry. Semi-purification of proteasomal complexes by gradient centrifugation from lumbar spinal cord of mutant SOD1 transgenic mice is not feasible because of tissue limitations. However, this method might be feasible by pooling lumbar spinal cords of mutant SOD1 transgenic rats, which are larger. In fact, recently a proteomic study of ER stress in lumbar spinal cord of pre-symptomatic rats were carried out by Atkin and colleagues (Atkin et al., 2006). Alternatively, the development of new antibodies against subunits of the 20S core to immunoprecipitate proteasomal complexes is necessary since the existing commercial antibodies are not suitable for these purposes. These antibodies are essential for characterization by mass spectrometric analysis of post-translational modifications in proteasomal subunits in ALS and other neurodegenerative disorders.

Finally, more biophysical and biochemical studies in various physiological contexts are needed to understand the biogenesis, formation of the structre, and complex activity of the proteasomal species at a cellular, tissue, organ, and organism level. Through these studies it would be possible to understand how the decrease of certain subunits at the protein levels observed during ALS pathogenesis can correspond to changes in proteasome activity. Interestingly, the half-life of certain subunits could be modified due to post-translational modifications. *In vitro* studies of the half-life of proteasomal subunits in cell lines treated with proteasomal inhibitors or overexpressing mutant SOD1 could indicate some of the changes that might occur *in vivo*.

The studies in spinal cord tissue of sporadic ALS patients demonstrate that there is a focal proteasome dysfunction. Immunohistochemical analysis with specific antibodies in spinal cord sections of these patients is required to determine if there are changes in localization of proteasomal subunits and whether loss of proteasomal subunits occurs in motor neurons of sporadic ALS patients, as documented in animal models of fALS. A recent screen of gene expression in laser captured motor neurons of sporadic ALS patients compared to controls by gene array did not identify proteasomal subunits. However, these genes are not well represented on the array that was used (Jiang et al., 2005). It would be important to assess specifically levels of mRNA expression by rt-PCR using specific primers for various subunits of the 20S core. Finally, assembly of 20S and 26S proteasome complexes and post-translational modifications of proteasome subunits should be determined in spinal cord tissue obtained from post-mortem sporadic ALS patients. These studies are necessary to determine the mechanisms that lead to impairment of proteasome activity in sporadic ALS patients and how these mechanisms might be different from results derived from animal models of fALS prior to designing therapies to target the ubiquitin-proteasome pathway to treat ALS patients.

Even though research describing physiological and pharmacological drugs to boost the ubiquitin-proteasome pathway and/or relieve proteasomal inhibition is still limited, several avenues of research to directly target this pathway to treat ALS already exist. Nrf2, a transcription factor that regulates the expression of ARE genes (see Section 1.2.1) has also been demonstrated to up-regulate gene expression of several subunits of the 20S core (Kwak et al., 2003b). Several chemical agents that lead to up-regulation of Nrf2 such as 3H-1,2-dithiole-3-thione (D3T) and tert-butylhydroquinone (tBHQ) are known. Treatment with tBHQ decreased toxicity that astrocytes purified from $SOD1^{G93A}$ transgenic rats exerted on motor neurons possibly by up-regulating glutathione and decreasing oxidative stress in the astrocytes (Vargas et al., 2006). Treatment of animal models of fALS with 3H-1,2-dithiole-3-thione

(D3T), a drug known to protect against a variety of chemical carcinogens and up-regulate Nrf2, specifically in lumbar spinal cord might slow down disease progression by decreasing levels of oxidatively damaged proteins as well as increasing levels of proteasome expression in tissue affected by the disease.

Other studies should be carried out to assess whether overexpression of a key ubiquitin-proteasome subunit could increase proteolytic capacity and lower mutant SOD1 toxicity. Since overexpression of 20S β5 in human fibroblasts promotes the expression of other 20S subunits and *de novo* formation of proteasomes, it will be necessary to determine whether overexpression of this subunit in murine primary spinal cord leads to increased proteasome activity and protein levels of other 20S subunits. One experiment would be to express this subunit in motor neurons from primary spinal cord cultures by microinjection of plasmid expression vector and determine its effect on expression of other proteasomal subunits and toxicity of $SOD1^{G93A}$. Similar studies could be conducted to evaluate protective properties of the E3 ligases, CHIP and dorfin which have been demonstrated to ubiquitinate mutant SOD1 *in vitro*. These studies would further establish the ubiquitin-proteasome pathway as a causative factor in ALS pathogenesis.

Chapter 8 Reference List

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and damaged proteins might contribute to their vulnerability to disease. We have been studying how mutations in the enzyme, Cu/Zn-superoxide dismutase (SOD1) cause a familial form of amyotrophic lateral sclerosis (ALS). We showed that proteasome function is disrupted specifically in lumbar spinal cord of G93A SOD1 transgenic mice, the tissue that will eventually be most affected in the disease. In the renewal of funding for this project, we propose to identify the mechanisms responsible for the tissue-specific loss of proteasome activity and the loss of proteasomes in motor neurons that precedes their death, and to determine if treatments that increase expression of protein chaperones prevent proteasome dysfunction.

4. Has an unanticipated problem occurred?	YES ☐ NO ☒ if yes, supply details:

5. If creating genetically modified animals or new combinations of genetic modifications, complete and attach a *Phenotype Disclosure form* (http://www.mcgill.ca/rgo/animal/forms/)

6. Procedures

a) For B and C level of invasiveness,

The procedures are the same as the original protocol: YES☒ NO☐

IF NO, complete the following:
Detail new procedures that are different from section 10a of the original protocol *(include a copy of the entire revised procedure section 10a of the original protocol with the changes and/or new procedures in CAPS):*

b) For D level of invasiveness,

Include here ALL procedures except transgenic procedures, including the ones described in the original protocol as well as new and changed procedures in CAPS *(was section 10a in main protocol); Please only attach SOPs related to new and changed procedures to this renewal form.*

7. Endpoints

a) For B and C level of invasiveness,

The procedures are the same as the original protocol: YES☒ NO☐

IF NO, supply new endpoints that are different from the original protocol:

b) For D level of invasiveness,

Include here ALL endpoints, including the ones described in the original protocol as well as new and changed endpoints in CAPS:

8. Hazards (check here if none are used: ☒)

a) Are the hazards different from original protocol? (infectious, radioactive, toxic, carcinogen, tumours)

YES ☐ NO ☐ if yes, supply details (material, risks, precautions):

b) Have the cell lines been tested for human and animal pathogens? YES:☐ NO:☐ None used:☒

9. Description of Animals to be used in the coming year (only):

Quality Control Assurance: To prevent introduction of infectious diseases into animal facilities, a health status report or veterinary inspection certificate may be required prior to receiving animals from all non-commercial sources or from commercial sources whose animal health status is unknown or questionable. Quarantine and further testing may be required for these animals. *If more than 6 columns are needed, please attach another page.*

	Sp/strain 1	Sp/strain 2	Sp/strain 3	Sp/strain 4	Sp/strain 5	Sp/strain 6
Species	mouse	mouse	mouse	mouse	mouse	mouse
Supplier/Source	Charles River					Taconics
Strain	CD1	B6SJL-Tgn-G1 mutant SOD1	nontransgenic littermates from G1 line	B6SJL-Tgn N29 wild type SOD1	nontransgenic littermates from N29 line	B6SJL breeding stock
Sex	F	F&M	F&M	F&M	F&M	F
Age/Wt	E13 gestation					5-6 weeks
# To be purchased	50					25
# Produced by in-house breeding		75	75	75	75	
# Other (e.g. field studies)						
TOTAL#/YEAR	**50**	**75**	**75**	**75**	**75**	**25**

10. Justification of Animal Numbers:

BASED ON THE EXPERIMENTAL OBJECTIVES OF THE PROJECT, describe the number of animals required for one year. Include information on experimental and control groups, # per group, and failure rates. For breeding, specify how many adults are used, number of offspring produced, and how many offspring are used in experimental procedures. The arithmetic explaining how the total of animals for each column in the table above is calculated should be made clear.

Strain 1: CD1 mice are used to prepare primary cultures of spinal cord for microinjection of expression vectors and testing of drugs for induction of HSPs. Spinal cord cultures are prepared from 13 day embryos and used at 4-8 wks in vitro. Embryonic animals are required at this age since motor neurons cannot be cultured from more mature animals. Generally preparation of cultures from two litters every two weeks provides a constant supply of cultures at the appropriate age for use in experiments (50 animals).

Transgenic mice:
Strain 6: Approximately 25 B6SJL female breeders are purchased from Taconics. Breeding as for SOP. [Note, these breeders are also used to generate mice listed in protocol #4610.]
Litters of mice are required for 3 time points (e.g. 45, 60 and 75 days of age) X 5 animals per assay X 5 assays = 75 mice per line. [Assays: characterization of modifications to mutant and wild type SOD1; effect of variants on proteasome activity; composition of proteasomes; mRNA levels for proteasome subunits in tissue; immunohisotochemistry.] This would require 25 matings for each of strains 2 and 4 (male hemizygote mated to nontransgenic female) according to the following calculation: with an average of 6 offspring per litter, 150 progeny would be generated of which 50% or 75 would be estimated to be transgenic for each of the mutant

SOD1 and wild type SOD1 lines. For each line, 75 non-transgenic mice would be generated and used as littermate controls (strains 3 and 5).
Unsuitable products of breeding are euthanized (i.e., not required for study or breeding stock).

Submit to your local Facility Animal Care Committee. Please note that after two renewals, a full protocol needs to be submitted.

This approval does not imply that space will be made available. If a major increase of space needs is anticipated, please contact the appropriate animal facility manager.